S-Nitrosothiols, nitric oxide and platelet and vascular dysfunction in cirrhosis

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Acknowledgements

My thanks to Professor Kevin Moore for the opportunity to undertake this work, for his expert guidance throughout and allowing me the freedom to follow my own directions. Thanks also to Dr David Harry and Dr Alireza Mani for their support and their time and to the Sir Jules Thorn Trust for funding this project. Finally, my thanks to my family for their constant love and support.

Abstract

Elevated plasma concentration of the known platelet inhibiting species S-nitrosothiols are correlated for the first time to platelet dysfunction in the bile duct ligated (BDL) cirrhotic rat. Further, a BDL model of cirrhosis has been established and characterised in a strain of analbuminaemic rats and a correlation made between a protection against S-nitrosothiol formation conferred by analbuminaemia and less dysfunction in platelet aggregation following induction of endotoxaemia and cirrhosis.

Also, it has been demonstrated that the majority of signal previously attributed to S-nitrosothiols during chemiluminescent detection in the plasma from endotoxaemic cirrhotic rats is mercury stable and is therefore attributed to N-nitrosamine formation. The identity of the mercury stable fraction of NO carrying species in plasma has been partially characterised and found to be carried either on albumin or on an albumin associated molecule. It has also been established that when the conjectured N-nitrosamine is synthesised *in vitro* and incubated with platelets, it is found to have an inhibitory effect on platelet aggregation similar to that found from S-nitrosothiols.

Hypo-reactivity of isolated aortic rings to vasoconstrictors has also been confirmed in the rat model of BDL cirrhosis. However, the injection of cirrhotic rats with lipoic acid (a thiol antioxidant shown to ameliorate the development of the hyperdynamic circulation in

the bile duct ligated rat), had no effect on the reactivity of isolated aortic rings as was initially hypothesised.

Finally, an adaptation of the Ellman's reaction for analysis of total reduced thiol concentration in plasma was validated and used to assess plasma reduced thiol concentration in cirrhotic rats. It was found that chronic, 25 day bile duct ligation results in a drop in free thiol concentration in the plasma of 75% over sham operated rats.

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12-HETE	**12-hydroxyeicosatetraenoic acid**
1°	**Primary**
^{3}H	**Tritium**
AA	**Arachidonic acid**
ACh	**Acetylcholine**
ADP	**Adenosine diphosphate**
ALT	**Alanine aminotransferase**
AMP	**Adenosine monophosphate**
ApoE	**Apolipoprotein E**
APTT	**Activated partial thromboplastin time**
AST	**Aspartate aminotransferase**
BDL	**Bile duct ligation (model of cirrhosis)**
BH4	**Tetrahydrobiopterin**
BSA	**Bovine serum albumin**
Ca^{2+}	**Calcium ion**
CaCM	**Calcium-Calmodulin**
cAMP	**Cyclic AMP**
CCl_4	**Carbon tetrachloride (model of cirrhosis)**
CFV	**Cyclic flow variation**
cGMP	**Cyclic guanosine monophosphate**

CI	**Cardiac Index**
DEA/NO	**Diethylenetriamine pentaacetic acid**
DHLA	**Dihydrolipoic acid**
DMSO	**Dimethylsulphoxide**
DNA	**Deoxyribonucleic acid**
DTNB	**Dithionitrobenzene**
DTPA	**Diethylene diamine tetra acetate**
DTT	**Dithiothreitol**
EDRF	**Endothelium Derived Relaxation Factor**
EDTA	**Ethylene diamine tetra acetate**
eNOS	**Endothelial nitric oxide synthase**
FAD	**Flavin adenine dinucleotide (oxidised)**
Fe	**Iron**
FMN	**Flavin mononucleotide (oxidised)**
g	**Gram**
g	**Gravity**
GFR	**Glomerular filtration rate**
GLC-MS	**Gas-liquid chromatography and mass spectrometry**
GSH	**Glutathione**
GSNO	**S-nitrosoglutathione**
GTN	**Glyceryl trinitrite**
GTP	**Guanosine triphosphate**
H^+	**Hydrogen ion**
H_2O_2	**Hydrogen peroxide**

Hb	**Haemoglobin**
Hb-NO	**Nitrosyl-haemoglobin**
HDL	**High density lipoprotein**
Hg^{2+}	**Mercury (II) ion**
HgSNOCA	**Mercury-stable NO-carrying albumin**
HgSNOCS	**Mercury-stable-NO-carrying species**
HgSNP	**Mercury-stable nitrosate plasma**
HMW	**High molecular weight**
HRS	**Hepatorenal syndrome**
HSA	**Human serum albumin**
I.P.	**Intraperitoneal**
IL-6	**Interleukin-6**
INF	**Interferon**
iNOS	**Inducible nitric oxide synthase**
KDa	**Kilodaltons**
L	**Litre**
LA	**Lipoic acid**
LDL	**Low density lipoprotein**
LMW	**Low molecular weight**
L-NAME	**N-nitro-L-arginine methyl ester**
LPS	**Lipopolysaccharide**
M	**Molar**
MAP	**Mean arterial pressure**
MAP	**Mean arterial pressure**

mL	**Millilitre**
mM	**Millimolar**
MQ	**milliQ water**
mRNA	**Messenger RNA**
MtNOS	**Mitochondrial nitric oxide synthase**
NAC	**N-acetylcysteine**
NAD^+	**Nicotinamide adenine dinucleotide (oxidised)**
NADH	**Nicotinamide adenine dinucleotide (reduced)**
$NADP^+$	**Nicotinamide adenine dinucleotide phosphate (oxidised)**
NADPH	**Nicotinamide adenine dinucleotide phosphate (reduced)**
NAPQI	**N-acetyl-ρ-benzoquinone**
NAR	**Analbuminaemic**
NEM	**N-ethylmaleimide**
nM	**Nanomolar**
nNOS	**Neuronal NOS**
NO	**Nitric Oxide**
NO^+	**Nitrosonium ion**
NO_2	**Nitrogen dioxide**
NO_2^-	**Nitrite**
NO_3^-	**Nitrate**
NOA	**Nitric oxide analyser**
NOS	**Nitric oxide synthase**
NOx	**Nitrite+nitrite**
O_2	**Oxygen**

O_3	Ozone
ODQ	[1H-[1,2,4]oxadiazolo-[4,3,-a]quinoxalin-1-one
Oxy-Hb	Oxidised haemoglobin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
PDTC	Pyrrolidine dithiocabamate
PE	Phenylepherine
PGI_2	Prostacyclin
PP	Portal Pressure
PRP	Platelet rich plasma
PT	Prothrombin time
RAAS	Renin-angiotensin-aldosterone-system
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RS	Reduced sulphydryl
$RS^{\cdot}$	Thiyl radical
RSNO	S-nitrosothiol
RT	Room temperature
RT-PCR	Real time PCR
S.C.	Subcutaneous
sGC	Soluble guanylate cyclase

SNAP	**S-nitroso-N-acetylpenecillamine**
SNO-4B	**Sepharose-4B-linked S-nitrosoglutathione**
SNO-albumin	**S-nitrosoalbumin**
SNO-Hb	**S-nitrosohaemoglobin**
SNP	**Sodium nitroprusside**
SNS	**Sympathetic nervous system**
SpD	**Sprague Dawley**
SVR	**Systemic vascular resistance**
TNF	**Tumour necrosis factor**
TXA_2	**Thromboxin A_2**
UV	**Ultraviolet**
vWF	**Von Willebrand Factor**
WP	**Washed platelets**
WT	**Wild type**
X-NO	**Unknown NO-carrying species**
all	**Microlitre**
μM	**Micromolar**

1. Introduction

1.1. Nitric oxide

Nitric oxide (NO) is a gaseous free radical with a short half-life. It has an unpaired electron and is involved in cell signalling and physiological regulation of vascular tone and platelet function. The discovery that NO is produced endogenously and accounts for the activity of the previously elusive "Endothelium Derived Relaxation Factor" (EDRF) won Furchgott, Murad and Ignarro the Nobel Prize for Physiology and Medicine in 1998.

1.1.1. Formation of nitric oxide (nitric oxide synthase)

NO is formed when nitric oxide synthase (NOS) converts L-arginine into L-citrulline in an O_2 and NADPH dependent reaction (Figure 1).

Three isoforms of the NOS enzyme have been identified plus a putative mitochondrial NOS isoform. Of the three isoforms of NOS identified NO is produced constitutively by eNOS (endothelial NOS) and nNOS (neuronal NOS), whereas the activity of iNOS (inducible NOS) is induced by a variety of factors, including lipopolysaccharide (LPS) and various cytokines. The mitochondrial (mtNOS) isoform appears to be a sub-isoform of nNOS. All 3 enzymes are dimers with 2 calmodulins (CaCM) attached and contain the tightly bound cofactors tetrahydrobiopterin (BH_4),

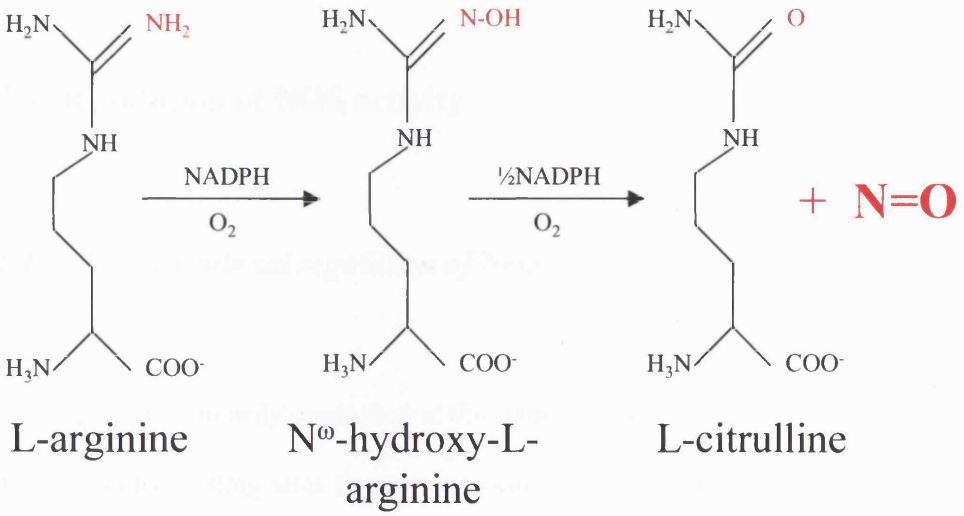


Figure 1. Nitric oxide synthase (NOS) catalysed synthesis of nitric oxide: NO is synthesised in the conversion of L-arginine to L-citrulline in an oxygen and NADPH dependant reaction

Flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN) and haem. The two constitutively expressed isoforms, eNOS and nNOS, are calcium dependent, whereas iNOS, although CaCM associated, is relatively insensitive to changes in Ca^{2+} concentration.

1.1.1.1. Regulation of NOS activity

1.1.1.1.1. Transcriptional regulation of NOS

The iNOS gene is primarily controlled at the transcriptional level. The promoter region of the gene contains binding sites for transcription factors including nuclear factor-kappa B (NFκB) and signal transducer and activator of transcription 1 (STAT-1). Lipopolysaccharide (LPS) and interferon-gamma (INF-γ) are key mediators of iNOS induction with LPS mediating pro-inflammatory responses through the NFκB pathway[1] and INF-γ through STAT-1[2]. NFκB induction requires the disassociation of NFκB from its associated inhibitory protein IκB through ubiquitination and phosphorylation of IκB[3].

There is a feedback regulation of iNOS activity mediated by NO itself. In 8hr. cytokine-treated rat renal mesangial cells, iNOS transcription is lower than in untreated cells, but incubation with NO donors increased iNOS transcription 3-fold[4]. Time-course studies reveal that NO donors first induce but then decrease iNOS transcription in a biphasic manner. This effect is also observed upon incubation with cGMP analogues[4]. In

hepatocytes, iNOS induction is regulated by NO through NO-mediated inhibition of IκB phosphorylation[5]. However, NO has also been shown to upregulate iNOS activity under certain conditions. For example, when hepatocytes are incubated with SNAP (an S-nitrosothiol which releases NO) for 8 hours and then allowed to recover in fresh medium for 24 hours, they subsequently exhibit increased NO synthesis over control cells[6]. This appears to be mediated by NO induction of tetrahydrobiopterin (BH_4) synthesis, an essential cofactor for NOS dimerisation and therefore increased iNOS dimerisation with unaltered iNOS expression.

The activation of NFκB is also under redox control. Thus, LPS-induced hydrogen peroxide (H_2O_2) production up-regulates iNOS expression in macrophages through activation of NFκB[7]. Administration of various antioxidants inhibits activation of NFκB. However, although in these experiments the antioxidant functions as a scavenger of ROS, studies have also shown that antioxidants seem to lower NFκB activity by direct mechanisms as well. Thus, N-acetylcysteine (NAC) has been shown to lower TNF-receptor affinity for TNF, and pyrrolidine dithiocabamate (PDTC) has been shown to inhibit IκB-ubiquitin ligase activity, both of which are independent of ROS production[8]. There has been interest in the use or role of thiol-based antioxidants in the regulation of NFκB/iNOS activity. An *in vivo* study in rats has shown that, whilst NAC can prevent the induction of iNOS and decrease NO synthesis in peripheral blood cells, it has no effect on iNOS activity once it is induced, indicating that NAC affects iNOS expression rather than activity[9].

1.1.1.1.2. Calcium/Calmodulin

Calmodulin is necessary for the activity of all 3 isoforms of NOS. The difference in iNOS and the constitutive NOS requirement for CaCM has been demonstrated to be a 40-50 amino acid insert in middle of the FMN binding sub-domain present in nNOS and eNOS but missing in iNOS[10]. This insert appears to de-stabilise the binding of CaCM to the nNOS and eNOS enzymes and explains their dependence on CaCM concentration[11,12]. CaCM concentration therefore regulates the activity of nNOS and eNOS enzymes but not that of iNOS.

1.1.1.1.3. Caveolin

The localisation of eNOS to the caveolae appears to be critical to the regulation of its activity. It is not certain whether or not eNOS is solely located in the caveolae or is present in other plasma membrane regions due to inconsistencies in results using different isolation methods[13,14]. Within the caveolae, eNOS associates with caveolin-1 via its scaffolding domain (amino acids 82-101), which inactivates the eNOS enzyme[15,16]. Activation of eNOS is signalled by an increase in cellular calcium concentration following receptor activation by acetylcholine[17], estradiol[18] or a calcium ionophore[17] or through the presence of shear stress[19]. An increase in intracellular calcium leads to the association of CaCM with eNOS and the displacement of caveolin-1. The release of

eNOS from the plasma membrane into the cytosol leads to its activation and enables NO synthesis[20,21].

1.1.2. Nitric oxide metabolism

NO has a short half life, being 5.6 seconds at atmospheric pO_2 but decreasing to 3.8 seconds after pO_2 is increased to 700mmHg[22]. The half-life of NO, however, is shorter *in vivo*. Thus, in a study in perfused isolated heart, the half-life of NO was calculated to be 100 ms through the coronary circulation[23]. Ultimately, the majority of NO is oxidised to nitrite and nitrate. The measurement of tissue, cellular and plasma concentrations of the stable NO metabolites, nitrite and nitrate, are therefore often used as an index of NO synthesis.

1.2. S-Nitrosothiols

1.2.1. Formation of S-nitrosothiols

1.2.1.1. Chemical synthesis

S-Nitrosothiols can be readily synthesised from the reaction of a thiol residue exposed to acidified nitrite

Equation 1). The reaction occurs with low molecular weight thiol molecules such as glutathione or cysteine[24] as well as proteins, such as albumin[25]. S-Nitrosothiols can also be formed from trans-nitrosation reactions in which one S-nitrosothiol transfers its –NO group to another thiol residue (see section 1.2.3.2). This reaction is particularly important in the synthesis of protein S-nitrosothiols. Not only does acidification introduce conformational changes to the protein, but it may also expose and reduce internal disulphide bridges. This could lead to S-nitrosation of residues unavailable to attack in the native protein.

$$NaNO_2 + HCl \rightarrow NaCl + HNO_2$$

$$HNO_2 + H^+ \rightarrow NO^+ + H_2O$$

$$RSH + NO^+ \rightarrow RSNO + H^+$$

Equation 1. Acidified nitrite mediated S-nitrosation of thiols.

Exposure of plasma to NO donors such as the NONOate family of NO donors has also been demonstrated to result in S-nitrosothiol generation[44,43]. However, the yield of S-nitrosothiol formed by this method is only about 10% of starting NO compound compared to >80% by transnitrosation from low molecular weight S-nitrosothiols[44] and is oxygen dependent[43].

1.2.1.2. Endogenous S-nitrosothiol formation

S-Nitrosothiol formation is not simply the addition of NO to a thiol group. In fact, the exact chemistry of S-nitrosothiol formation *in vivo* is not completely understood. It is accepted that in order for a reduced thiol to form an S-nitrosothiol bond directly it must react with the nitrosonium ion (NO^+) or an NO^+ donating species rather than NO. Many researchers have focused on the nitrogen oxides formed from the oxygen dependent pathway of NO metabolism in identifying a possible nitrosating molecule (see Equation 2).

$$2NO + O_2 \rightarrow 2NO_2$$

$$NO_2 + NO \leftrightarrow N_2O_3$$

$$2NO_2 \leftrightarrow N_2O_4$$

$$N_2O_4 + HS^- \rightarrow X\text{-}NO + NO_3^-$$

$$N_2O_3 + HS^- \rightarrow X\text{-}NO + NO_2^-$$

Equation 2. Oxygen dependent metabolism of NO.

It is widely believed that N_2O_3 or N_2O_4, both of which can donate NO^+, are the most likely nitrosating molecules *in vivo*. Kharitonov et.al.[26] carried out a study into the kinetics of S-nitrosothiol formation and found it to be oxygen dependent, first order to oxygen concentration and second order to NO concentration. The authors concluded that N_2O_3 is therefore the most likely nitrosating species. There has been comment that N_2O_3 is a highly unstable molecule in aqueous solution and that it would not exist long enough to come into contact with a thiol residue and react to form an S-nitrosothiol. However, as both NO and O_2 are relatively hydrophobic molecules, it has been suggested that these molecules are more stable and react more efficiently to form S-nitrosothiols in hydrophobic pockets of proteins, notably albumin as well as at the membrane interface[27],[28]. Direct S-nitrosation of reduced thiol residues therefore appears to be an oxygen dependent process.

However, S-nitrosothiols can be formed under anaerobic conditions. As only small traces of oxygen will react with NO to form higher nitrogen oxides, it is experimentally difficult

to totally isolate anaerobic nitrosation reactions. It has, however, been shown that NO can itself interact with thiols *in vitro* under basic conditions when oxygen is totally excluded, probably through reaction with the thiolate anion resulting in the formation of a disulphide[29] (Equation 3). This does not, however, appear to be nearly as efficient as oxygen-dependent nitrosation reactions and as such is less likely to account for the majority of endogenous S-nitrosothiol formation.

$$RSH + B^- \leftrightarrow RS^- + BH$$

$$RS^- + NO \rightarrow RS\text{-}N\text{-}O^- \leftrightarrow RS\text{-}N\text{-}OH$$

$$2RS\text{-}N\text{-}OH \rightarrow RSN(OH)N(OH)SR \rightarrow RSSR + N_2 + N_2O$$

Equation 3. Anaerobic nitrosation of thiolate anion.

There are also reports of S-nitrosothiol formation from exposure of thiols to peroxynitrite[30,31].

1.2.2. Stability of S-nitrosothiols

S-Nitrosothiols are sensitive to photolytic[32,123,124], ascorbate[33], transition metal ion[34], and thiol[33] mediated decomposition. Temperature and pH also affect their rate of decomposition[34]. S-Nitrosothiols synthesised in buffer *in vitro* are relatively stable if stored with a metal ion chelator[35].

1.2.3. Release of nitric oxide from S-nitrosothiols

The release of NO from S-nitrosothiols has been studied in biological fluids, and the rate of release has been found to be dependent on the S-nitrosothiol species, the plasma thiol concentration, ascorbate and the availability of transition metal ions.

1.2.3.1. Photolysis and transition metal stability

The only mechanism by which NO release is mediated by the homolytic cleavage of S-nitrosothiol bond, liberating ·NO and thiyl radical (RS·), is photolytic cleavage[36]. Metal ion-mediated release of NO results in the formation of NO and disulphide[37] or thiolate anion (reduced sulphydryl) but does not proceed through a thiyl radical intermediate[35,38].

$$2GSNO + Cu^{2+} \rightarrow GSSG + Cu^{2+} + 2NO$$

$$GSNO + Cu^{+} \rightarrow GS^{-} + NO + Cu^{2+}$$

Equation 4. Copper mediated NO release from S-nitrosoglutathione.

It appears that the copper(I)-mediated release of NO may be highly important in the action of S-nitrosothiols *in vivo*. Specific Cu^{+} chelators have been shown to inhibit the

vasodilatory response to GSNO and SNAP[39] and the antiplatelet effect of S-nitrosothiols[40].

1.2.3.2. Transnitrosation reactions

There is a dynamic interplay between S-nitrosothiols and free thiol groups and this is termed transnitrosation.

$$RSNO + R'SH \leftrightarrow RSH + R'SNO$$

Equation 5. Transnitrosation: transfer of nitroso-group from nitrosothiol to thiol.

It is a reversible process and involves the nucleophilic attack of the thiolate anion on the nitrogen of an S-nitrosothiol[41]. The relative reactivity of a thiol residue to attack an S-nitrosothiol is related to its pKa and concentration of thiol[42].

1.2.3.2.1. Interaction of protein and low molecular weight thiols and S-nitrosothiols

Protein S-nitrosothiols are generally much more stable than low molecular weight S-nitrosothiols. However, whilst the dynamic interplay that occurs *in vitro* has been extensively studied, the role *in vivo* is not clear.

Scorza *et.al.*[33] show that there is a 3-fold higher NO release from 200μM S-nitrosoglutathione (GSNO) when incubated with human plasma compared with an equimolar amount of S-nitrosoalbumin. Likewise, Marley *et.al.*[43] report a two-times higher rate of S-nitrosothiol disappearance when GSNO is incubated with human plasma than when SNO-albumin is incubated in human plasma and S-nitrosoalbumin is clearly more stable than low molecular weight S-nitrosothiol in plasma (half life S-nitrosoalbumin in plasma ~60mins vs. GSNO ~7mins).

The presence of low molecular weight thiols has a major impact on the decomposition of S-nitrosothiols. Thus, treatment of plasma with NEM (a thiol-blocking agent) enables the stabilisation of S-nitrosoalbumin and S-nitrosoglutathione in plasma. Further addition of exogenous glutathione to plasma rapidly accelerates S-nitrosoalbumin decomposition. The role of low molecular weight thiols was inferred indirectly by the observation that S-nitrosoalbumin had improved stability in plasma which had been dialysed against buffer compared to normal plasma. When GSNO is added to plasma, and the concentration of

protein S-nitrosothiols measured, between 30%[43] and 80%[44] of added GSNO can be recovered as S-nitrosated proteins.

When transnitrosation reactions are initiated in whole blood by incubation with low molecular weight (LMW) S-nitrosothiols, it is found that around 60% of added LMW S-nitrosothiols can be recovered as S-nitrosoalbumin and 20% as nitrite/nitrate. No LMW S-nitrosothiols could be recovered[44]. However, when the NO donor, detaNONOate is incubated with whole blood at 37°C, 2hr, relatively small amounts of S-nitrosothiol are formed[43], presumably because of scavenging of NO by erythrocytic haemoglobin.

1.2.3.2.2. Transfer of S-nitrosothiol activity across membranes

The mechanism of transfer of the nitrosonium anion (NO^+) from a circulating S-nitrosothiol to a cellular target in now thought to involve cell surface thiols in a mechanism involving protein disulphide isomerase (PDI). Located primarily to the endoplasmic reticulum, PDI has been shown to facilitate protein folding through two critical cysteine residues in its active site[45]. However, it has also been shown to be located to the plasma membrane of various cell types including platelets[46], endothelial cells[47] and exocrine pancreatic cells[48]. Disruption of PDI expression in a human erythroleukemia (HEL) cell line using antisense DNA to disrupt PDI mRNA translation was successfully achieved and resulted in a 74±9% decrease in cell surface PDI expression and a 53±2% decrease in cell surface thiol concentration[49]. Co-incubation of

isolated PDI with a molar excess of S-nitrosothiol SNO-4B (sepharose-4B-linked S-nitrosoglutathione) resulted in a concentration dependent transnitrosation of 2 NO molecules per PDI protein implying nitrosation of the two active site cysteines on PDI (see Figure 2). When the PDI-disrupted HEL cell line was incubated with 50μM SNO-4B, it was found that intracellular cGMP concentration was 65±27% lower than in control cells and 77±15% lower when incubated with 1μM SNO-albumin. A control experiment was also conducted in which intracellular cAMP concentration was measured after incubation with PGE_1 and was found to be slightly higher in PDI disrupted cells than in control cells. Ramachandran et.al. have gone on to confirm PDI-mediated transfer of (NO^+) from S-nitrosoalbumin across the human umbilical endothelial cell membrane using a fluorogenic substrate N-dansyl-S-nitrosohomocysteine (DnsHCys) (S-nitrosation abolishes the fluorescence of DnsHCys)[50]. The role of PDI in platelet aggregation has also been investigated and has been found to be involved in GSNO mediated inhibition of platelet aggregation by at least two mechanisms[51]. In addition to a role in transnitrosation ,PDI has also been shown to be critical for the binding of vWF to platelets[52].

Another mechanism of S-nitrosothiol transfer across the cell membrane has also been established from studies in cultured cells[53,54]. These studies show that S-nitrosocysteine transport across the cell membrane can be inhibited by the amino acid transporter system L (L-AT) inhibitor, 2-aminobicyclo[2.2.1]-heptane-2-carboxylate. Furthermore, these studies demonstrate that, whilst S-nitrosocysteine and S-nitrosohomocysteine can be directly taken up by cells, other S-nitrosothiols tested (S-nitrosoglutathione, SNAP and S-nitrosoalbumin) required conversion to S-nitrosocysteine for transport through the L-AT.

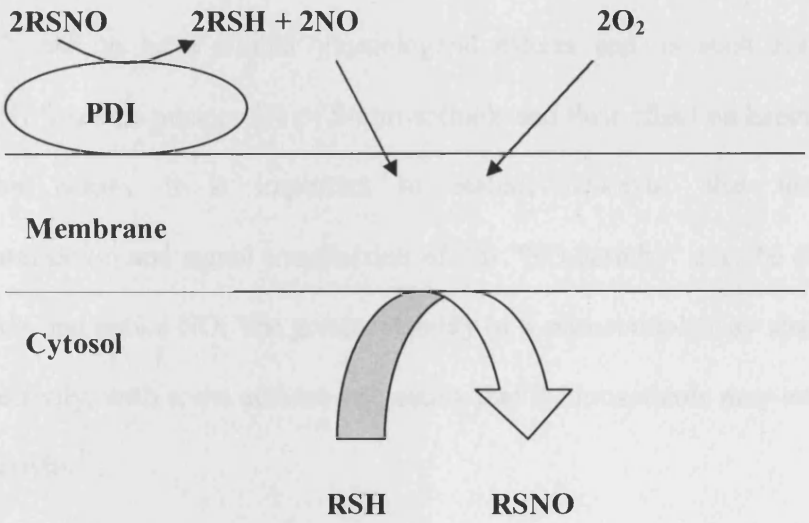


Figure 2. Proposed mechanism of protein disulphide isomerase (PDI)-mediated transfer of S-nitrosothiols (RSNO) across the plasma membrane. S-nitrosothiols react with cell surface PDI sulphydryl groups (RSH) liberating NO. NO then interacts with oxygen in the hydrophobic membrane environment forming nitrosating species such as N_2O_3. These higher nitrogen oxides then nitrosate intracellular sulphydryl groups resulting in transfer of S-nitrosothiol into the cytoplasm. Figure re-drawn from ref 50.

1.3. An important note on S-nitrosothiol and NO bioactivity

The widespread use of S-nitrosothiols as NO donors and the interaction of NO and thiols to form S-nitrosothiols makes the separation of native NO-mediated effects and S-nitrosothiol mediated effects difficult to separate. "Pure NO donors" and "S-nitrosothiol NO donors" tend to have similar physiological effects and as such are discussed predominantly from the perspective of S-nitrosothiols and their effect on haemostasis and vasodilatation below. It is important to realise, however, that the *in vivo* compartmentalisation and signal transduction of this "NO-activity" may be different for S-nitrosothiols and native NO. The greater stability of S-nitrosothiols may also be crucial for *in vivo* activity, with some authors suggesting that S-nitrosothiols may even account for EDRF activity[55].

1.4. NO, S-nitrosothiols and vasodilatation

1.4.1. NO, guanylate cyclase and cGMP

Soluble guanylate cyclase (sGC) is a haem-containing, cytosolic protein which catalyses the formation of cyclic guanosine monophosphate (cGMP) from guanylate triphosphate (GTP) [56]. Elevation of cGMP and activation of sGC are modulated by NO and NO

donors[57]. sGC activation by NO is mediated through binding of NO to the haem moiety of sGC[58].

1.4.2. cGMP and vasodilatation

Intracellular cGMP is increased after induction of vasodilatation in endothelium intact aortic rings by acetylcholine (ACh), calcium ionophore or NO donor compounds[59,60,61]. If the endothelium is removed then Ach-mediated vasodilation is abolished and cGMP concentration is not elevated in stimulated aortic rings[59]. However, endothelium denuded aortic rings still relax in response to nitroglycerin and the cGMP concentration rises concomitantly[59]. When cGMP concentration in ACh stimulated, endothelium intact aorta was analysed, it was found that most of the increase in cGMP was found in the smooth muscle rather than the endothelium[61]. Methylene blue, a guanylate cyclase inhibitor, was also shown to inhibit cGMP accumulation and relaxation in aortic rings[62]. These observations demonstrated that an endothelium-derived factor was responsible for Ach mediated vascular relaxation and that this factor caused activation of guanylate cyclase and production of cGMP which mediated smooth muscle relaxation. However, the identity of the endogenous modulator of guanylate cyclase activation was still unknown and was referred to as "Endothelium Derived Relaxation Factor" or EDRF.

1.4.3. Nitric oxide and EDRF

The fact that NO donating species elicit vasorelaxation through the same guanylate cyclase activating mechanism as EDRF led to investigations into whether NO could be EDRF. This hypothesis was confirmed in a study by Moncada et.al.[63] who used the chemiluminescent reaction of NO and ozone to measure NO release from cultured endothelial cells. Release of NO from the endothelial cells (but not smooth muscle cells) could be induced dose-dependently by bradykinin stimulation. The activity of EDRF (released from endothelial cells stimulated with 20nM bradykinin), 0.22nM NO gas and 50nM glyceryl trinitrite (GTN) was assayed using a cascade of aortic strips[64]. The activity of NO and EDRF was found to be identical: both caused relaxation in the aortic strips, an activity that diminished at the same rate as they progressed through the bioassay system (50% reduction in activity after 3.6±0.1seconds for EDRF and 4.1±0.2 seconds for NO). In contrast, GTN-induced vasorelaxation did not diminish as the GTN cascaded over successive aortic rings. The stability of EDRF and NO in Krebs buffer was determined by bioassay and found to be identical: 30.8±1.9 seconds and 30.4±2.2 seconds respectively. Scavenging of superoxide (which can react with NO to form peroxynitrite) by addition of superoxide dismutase (SOD) to the bioassay system resulted in equal prolongation of NO/EDRF activity. Addition of haemoglobin (a known NO scavenging species) to the bioassay system resulted in attenuation of the ability of both endothelial EDRF and NO to mediate relaxation. However, the IC_{50} of haemoglobin inhibition of relaxation was lower for EDRF than NO (3.6±0.6 and 8.1±1.4nM respectively). These observations were confirmed by Ignarro et.al.[65] in a perfused blood

vessel bioassay system who also went on to demonstrate the identical pharmacological profile NO and EDRF. They also showed that both EDRF released from aortic endothelial cells and NO react with haemoglobin to yield nitrosyl-haemoglobin.

1.4.4. Effect of NOS inhibitors on vascular tone

Infusion of a NOS inhibitor to the conscious rabbit results in vasoconstriction of the coronary, cerebral, renal and duodenal vascular beds[66]. Chronic infusion of a NOS inhibitor has actually been used as a model of hypertension in rats. Blood pressure rose from 108±3mmHg in controls to 164±6mmHg following 4-6 week inhibition of NOS with nitro-L-arginine[67]. It also led to renal vasoconstriction and a resulting 30% fall in glomerular filtration rate. Acute infusion of the NOS inhibitor L-NAME (50ug/Kg/min) to rats increases the mean arterial pressure (MAP) and lowers glomerular filtration rate (GFR) in rats[68]. When the cGMP analogue, 8-bromo-cGMP (200ug/Kg/min), is co-infused with L-NAME, no significant change in either MAP or GFR occur. NO synthesis, activation of guanylate cyclase and formation of cGMP is now known to be important in maintaining normal vascular tone. Vallance and Webb have also shown that infusion of the NOS inhibitor L-NG-monomethyl-arginine reduced forearm blood flow in human volunteers by 44%[69].

1.4.5. NO and S-nitrosothiol regulation of vascular tone

The role of NO and S-nitrosothiols in regulation of vascular tone has been extensively studied. The observation that inhaled NO has systemic effects led investigators to realise that the simple model of freely diffusing NO (½-life ~1-3secs) acting as a paracrine vasodilator cannot account for its total mechanism of action[70,71,72]. The prolongation of NO activity *in vivo* is particularly surprising as NO is known to interact rapidly with the haem group of deoxy-Hb to form an iron nitrosyl (Hb-NO) and to react with oxy-Hb to form methaemoglobin and nitrate[73]. Any mechanism which leads to stabilisation of NO activity would therefore have to out-compete these reactions. The nature of the stabilisation of NO activity has focused particularly on S-nitrosothiol formation.

Stamler and co-workers have suggested that erythrocytic haemoglobin (Hb) can allosterically bind and release NO from the haemoglobin thiol, cysteine β-93, forming S-nitroso-Hb (SNO-Hb) depending upon haemoglobin oxygenation state[74]. They suggest that oxy-haemoglobin has a greater affinity for NO than deoxy-haemoglobin so NO binds to cysteine β-93 in high oxygen tension and then releases NO in areas of low oxygen tension. The released NO causes vasodilatation and this leads to increased oxygen supply to the vascular bed with low oxygen tension. However, the reaction of NO with the cysteine β-93 would therefore have to overcome competition from haem NO scavenging in order to have physiological significance. Stamler et.al. provide evidence for increased binding of NO to cysteine β-93 (increased SNO-Hb formation) in high oxygen tension relative to formation of Hb-NO. However, this is widely disputed in other studies in

which different methodology is used to quantify erythrocyte SNO-Hb. Gladwin et.al. report only low nanomolar concentrations of SNO-Hb formed even after NO inhalation with Hb-NO being the predominant species formed[75]. Also, the studies in which the allosteric model of NO binding to haemoglobin were carried out used rat haemoglobin which has been shown to contain extra thiol residues rather than the one pair of thiols per tetramer of human haemoglobin[76]. Finally, recombinant human haemoglobin which have impaired ability to switch between oxy/deoxy states have been shown to elicit the same hypertensive effects as native haemoglobin[77].

The allosteric model of the haemoglobin/NO interaction in the literature appears to have fallen out of favour and other mechanisms of blood flow regulation have been proposed. Predominant amongst these theories is that nitrite, traditionally assumed to be an inactive metabolite of NO, may in fact be reduced *in vivo* and in fact be an NO storage molecule itself. This is discussed further in the section 1.6.3.

1.5. NO and S-nitrosothiols and platelet aggregation

1.5.1. cGMP dependent inhibition of platelet aggregation

The anti-platelet activity of NO and S-nitrosothiols was known prior to the identification of NO as EDRF[78]. Indeed, Loscalzo noted in 1985 that incubation of platelets with N-acetylcysteine and nitroglycerin potentiated the antiplatelet properties of nitroglycerin and correlated this to increased intraplatelet cGMP concentration[79]. Others have shown this increase in intraplatelet cGMP concentration following incubation with S-nitrosothiols[78,80,81]. Early studies looking at the effects of S-nitrosothiols on platelet aggregation considered only low molecular weight S-nitrosothiols, but in 1992 Stamler showed that protein S-nitrosothiols (albumin, tissue-type plasminogen activator and cathepsin B) can also be formed from proteins with a vicinal thiol residue and confer anti-platelet activity[82]. Studies have confirmed that chelation of copper using metal ion chelators reduces the anti-platelet activity of S-nitrosothiols[40,83]. Low molecular weight S-nitrosothiols have a greater, though shorter-lasting effect on platelet aggregation than protein S-nitrosothiols[83]. However, the co-incubation of low molecular weight thiols such as cysteine with protein S-nitrosothiol results in increased inhibition of platelet aggregation[83]. This seems to suggest transfer of NO^+ from S-nitrosoalbumin to low molecular weight thiols and the resulting formation of the corresponding low molecular weight S-nitrosothiol in the mechanism of S-nitrosoalbumin mediated inhibition of platelet aggregation. This is supported by data from Crane et.al. who show that addition of the NO donor DEA/NO to washed platelets (WP) and platelet rich plasma (PRP) elicits

different responses[84]. Incubation of DEA/NO with PRP and WP for 1 minute (during the period of NO release from DEA/NO) results in similarly impaired aggregation in both PRP and WP (82±5% and 91±2% inhibition respectively). However, after 30 minutes of incubation with DEA/NO (after which time no NO release from DEA/NO could be recorded), platelet aggregation in PRP is still impaired (72±7% inhibition) whereas that in WP almost completely returned to control levels (5±3% inhibition). However, if WP were then co-incubated with 1% serum albumin and exposed to DEA/NO for 30 minutes a significant decrease in platelet aggregation was observed (39±10% inhibition). If LMW thiols were also co-incubated the inhibition of platelet aggregation observed was restored to 1 minute DEA/NO incubation levels (>80% inhibition). LMW S-nitrosothiol intermediates therefore seem to be important in the action of S-nitrosoalbumin which is of distinct physiological relevance considering that the majority of S-nitrosothiols that can be detected in the circulation exist as S-nitrosoalbumin. It also raises the possibility that administration of LMW thiol into the circulation may decrease the circulating SNO-albumin reservoir.

It has been observed that co-incubation of S-nitrosothiols and either haemoglobin or red blood cells with platelets abolishes the anti-platelet activity of S-nitrosothiols[85]. This was shown to be due to the molar excess of haemoglobin (intra- or extra-cellular) scavenging the NO released from the S-nitrosothiol species in preference to guanylate cyclase activation. These authors conclude that this limits the potential of S-nitrosothiols as therapeutic anti-platelet agents. However, whilst this may be of pharmacological

significance, Kaposzta *et.al.* have successfully reduced the incidence of embolic signals by infusion of GSNO during carotid angioplasty surgery[86].

1.5.2. cGMP independent inhibition of platelet aggregation

Although the effect of NO-donors on platelet aggregation has been shown to signal through the classical cGMP pathway[87], it has recently been shown that there may be a cGMP-independent inhibitory action of NO donors on platelet aggregation. This observation was first noted by Gordge *et.al*[88] in human washed platelets who demonstrated that different NO donors elevated intraplatelet cGMP concentration in direct proportion to their rate of NO release (as detected by extracellular oxy-haemoglobin to met-haemoglobin accumulation). However, there was no correlation between the cGMP concentration/NO release from different NO donors and the degree of inhibition of platelet aggregation they elicited. DEANO released NO at a greater rate than GSNO and caused a greater rise in intraplatelet cGMP concentration than GSNO, but GSNO inhibited platelet aggregation to a greater degree. Furthermore, the guanylate cyclase inhibitor, [1H-[1,2,4]oxadiazolo-[4,3,-a]quinoxalin-1-one (ODQ), inhibited accumulation of platelet cGMP in response to GSNO incubation, but this only attenuated rather than abolished the observed inhibition of platelet aggregation elicited by GSNO. Finally the authors demonstrate that thiol concentration in the platelet membrane and the availability of copper are important for GSNO induced non-cGMP mediated inhibition of platelet aggregation.

Sogo *et.al.*[89] note a similar non-cGMP mediated inhibition of platelet aggregation in platelet rich plasma (PRP) using ODQ with several NO donors (GSNO, diethylamine diazeniumdiolate {DEA/NO}, RIG200 and glyceryl trinitrate {GTN}) but noted that sodium nitroprusside (SNP) induced inhibition of platelet aggregation was entirely cGMP dependent. This raises the question as to whether the nature of the NO release from the different NO donors determine the ratio of cGMP-dependent to cGMP-independent inhibition of platelet aggregation they elicit. When the authors measured NO release in PRP from each of the donor compounds studied (using an NO electrode) it was found that whilst NO release could be detected from DEA/NO, RIG200 and GSNO, there was no detectable NO release from SNP. This correlated with their observation that incubation of Fe(II)-haemoglobin with platelets prior to addition of the NO-donor compounds resulted in partial restoration of aggregation in platelets incubated with DEA/NO, RIG200 and GSNO but not with SNP. The authors therefore suggest that native NO release into the extracellular environment may be necessary for cGMP-independent inhibition of platelet aggregation. They speculate that as nitrosothiol NO-donors (NO^+ donors) have a greater inhibitory effect on ADP induced aggregation than DEA/NO ($^{\cdot}NO$ donor) and DEA/NO has a greater inhibitory effect on collagen induced aggregation, nitrosation of platelet ADP receptors and nitration of collagen/TXA_2 receptors may be an area for investigation of the mechanism of cGMP-independent inhibition of platelet aggregation.

There have been various mechanisms suggested to explain the cGMP-independent mechanism of platelet aggregation. Trepakova *et.al*[90]. and Wanstall *et.al.*[91] have shown I human and rat platelets respectively, that NO increases the rate of SERCA-dependent filling of Ca^{2+} stores and that inhibition of the SERCA-dependent filling of these stores with BHQ abolished these effects of NO. Crane *et.al* have also carried out experiments which show that extracellular release of NO radical is required for cGMP-independent inhibition of platelet aggregation. Oberprieler *et.al.*[92] have shown that GSNO inhibits the adhesion of GPIIIa/IIb to fibrinogen. The authors show that this effect of GSNO is associated to decreased phosphorylation of the β_3-integrin on tyrosines 773 and 785. The authors speculate that the underlying mechanism may be nitrosation/nitrtation of critical cysteine residues in a cysteine rich extracellular region of the β_3-integrin which are exposed during structural re-arrangements facilitating binding of fibrinogen to the itegrin.

1.5.3. Platelet NO synthase

Many studies have reported nitric oxide synthase activity in platelets, however, there is controversy as to which isoform or isoforms are expressed.

NO release from platelets during platelet aggregation has been detected directly using NO electrodes[93,94,95]. Further, administration of L-arginine to platelets increased NO synthesis and NOS inhibitors decrease the NO signal from platelets, confirming NO production through the L-arginine:NO pathway. However, one study reported basal NO release in unstimulated platelets by indirect measurement of intra-platelet nitrite and nitrate

accumulation and conversion of oxy-haemoglobin to met-haemoglobin; observations that could be abolished by administration of a NO synthase inhibitor[96]. Furthermore, incubation of L-arginine with human platelets inhibits platelet aggregation and NOS inhibitors enhance platelet aggregation in response to a variety of agonists[95,97,98]. However, whilst inhibition of NOS in human platelets leads to enhanced platelet aggregation, no effect has been demonstrated in rat platelets. Whilst many studies have shown infusion of NOS inhibitors to enhance platelet function in various pathological situations, the few studies that have included control experiments in which L-NAME is administered to normal rats has shown no change in platelet aggregation[99] or bleeding time[100].

Both iNOS and eNOS proteins and mRNA have been reported in human platelets. Chen et.al.[101] report iNOS and eNOS mRNA in platelet lysates by RT –PCR and Southern blotting and eNOS and iNOS proteins using monoclonal antibodies against each protein. Western blotting demonstrated that the protein extracted by the eNOS antibody had a molecular weight of 140-150kDa and iNOS a molecular weight of 200kDa. They also report that incubation of platelets with interferon-γ and LPS increased expression of iNOS protein. However, Sase et.al.[102] report only eNOS mRNA expression in platelets and could not detect mRNA using human iNOS or nNOS primers. Indirect evidence for both a constitutive NOS and iNOS in platelets comes from the study of Chen et.al[101]. They show that ^{3}H-L-arginine conversion to ^{3}H-L-citrulline can occur only when platelets are suspended in a Ca^{2+}-rich buffer but not when suspended in Ca^{2+}-free buffer suggesting a constitutive, Ca^{2+}-sensitive NOS enzyme. They then show that when

platelets are stimulated with LPS/cytokines in a Ca^{2+}-free buffer, an increase in ^{3}H-L-citrulline synthesis is observed, indicating induction of iNOS.

Experiments using eNOS knockout mice ($eNOS^{-/-}$) have shown the importance of platelet eNOS in control of platelet activation[103]. Firstly, the bleeding time in eNOS knockout mice has been shown to be significantly shorter than in wild type (WT) (77.2±3 seconds vs. 133.4±3 seconds). Furthermore, when platelets isolated from WT and $eNOS^{-/-}$ mice are injected into thrombocytopaenic eNOS knockout mice, it is found that the platelets infused from eNOS knockouts decrease the bleeding time to a significantly greater degree than platelets infused from wild type mice (Δbleeding time, -24.6±9.1 and -3.4±5.3 seconds, respectively).

1.5.4. Regulation of NOS activity in platelets

NOS activity in platelets is regulated by a variety of cofactors and agonists as well as by substrate bioavailability.

Tetrahydrobiopterin (BH_4) is an essential NOS cofactor, the availability of which regulates NO production from NOS. In a canine model of coronary arterial thrombosis which presented with cyclic flow variations (CFVs) it has been shown that intraplatelet BH_4 and cGMP levels were reduced compared to controls and platelet aggregation was enhanced[104]. Supplementation with BH_4 led to an increase in intraplatelet BH_4 and cGMP levels, decreased platelet aggregation and attenuation of CFVs. However, when BH_4 was

co-administered with a NOS inhibitor the end response, i.e. decreased platelet aggregation and attenuation of CFVs were observed.

Incubation of platelets with β-adrenoceptor agonists is known to inhibit platelet aggregation and the link between this effect and NOS induction has been examined. The study by Queen et.al.[105] shows that stimulation of β-adrenoceptors with isoproterenol increases intra-platelet NOS activity approximately twofold. This increase in NOS activity could also be induced by incubation of platelets with forskolin, an adenylate cyclase activator. When platelets were co-incubated with an adenylate cyclase inhibitor this increase in NOS activity was abolished. Functionally, the presence of a NOS inhibitor does not affect the inhibition of aggregation induced by isopterenol when aggregation is induced by a thromboxane analogue. However, NOS inhibitors attenuated isopterenol mediated inhibition of platelet aggregation in platelets stimulated with thrombin. This implies that β-adrenoceptor activation of the L-arginine/NO system regulates platelet adhesion rather than aggregation.

More recently, it has been shown that α-tocopherol can inhibit platelet aggregation and this is secondary to a marked increase in NO synthesis. There is some controversy over these data and the effect of γ-tocopherol[106,107], but all tocopherols have been shown to enhance NOS activity. Increased platelet eNOS phosphorylation was observed in tocopherol treated platelets, suggesting a possible mechanism of NOS activation[106]. However, as neither of these studies assessed the effect of NOS inhibitors, it is

impossible to tell definitively if the rise in NOS activity in these circumstances causes the observed inhibition of platelet aggregation.

Platelet NOS activity may also be regulated by the uptake of L-arginine by the platelet. Homocysteinaemia is an independent risk factor for atherosclerosis, thrombosis and other cardiovascular diseases. Reports have shown that homocysteine incubation can inhibit platelet uptake of L-arginine, decrease intraplatelet cGMP concentration and increase intracellular calcium concentration[108,109].

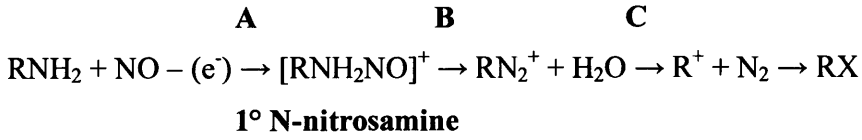
1.6. Other biologically relevant nitric oxide carrying molecules

1.6.1. Nitric oxide and iron proteins

NO can react directly with both ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron and this has been shown to occur in numerous iron-containing proteins, often affecting their function. NO mediated inhibition of iron-containing enzymes occurs predominantly in haem-containing proteins as the Fe-NO interaction is considerably more stable in this structure[110] than in other iron containing proteins. Among the haem proteins affected by NO are haemoglobin and the P450 cytochrome family of enzymes, as well as NOS itself. These interactions are discussed further in the context of their biological actions later.

1.6.2. N-nitrosation

N-Nitrosation describes the formation of N-nitrosamines and related compounds and involves the formation of an R-N-N=O bond. Aromatic and aliphatic primary and secondary amines can be N-nitrosated *in vitro* and tertiary amines have also been demonstrated to form N-nitrosamines, however, at much lower rate (10 000-fold slower than secondary amines)[111]. Primary N-nitrosamines are generally unstable, whereas secondary amines are highly stable.



Equation 6: Primary N-nitrosation (A). Primary nitrosamines are inherently unstable and decompose by diazotization (B) and deamination (C) reactions.

$$R_2NH + NO\ (-e^-) \rightarrow R_2NNO + H^+ \qquad \textbf{(A)}$$

$$NO_2^- + H^+ + R_2NH \rightarrow R_2NNO + H_2O \qquad \textbf{(B)}$$

Equation 7. N-nitrosation of secondary amines. (A) One electron oxidation of NO in the presence of secondary amines can result in high yields of secondary amines. (B) Acid-catalysed reaction of nitrite with secondary amines producing N-nitrosamines.

N-nitrosamines are carcinogenic *in vivo* and dietary intake is correlated to oesophageal and other gastrointestinal cancers[112]. Formation of N-nitrosamines can occur as part of the food preparation procedure, especially associated with the use of nitrite in the preparation of cured meats[113]. It is also possible to generate N-nitrosamines *in vivo* as the result of dietary ingestion of nitrite into an acidic environment (e.g. the stomach)[114]. Cigarette smoke also contains mutagenic N-nitrosamines[115].

1.6.3. O-nitrosation - nitrite

Nitrite has traditionally been considered an inactive, stable metabolite of NO metabolism *in vivo*. This view is based on studies in which nitrite applied to isolated vascular tissue only elicits vasodilatation in supra physiological concentrations[116,117]. However, recent studies have shown that infusion of nitrite at near-physiological concentrations can elicit vasodilatation as determined by increased blood flow[118] and this is dependent on deoxy-haemoglobin which acts as a nitrite reductase. The vasodilatation induced by nitrite is dependent on oxygen tension[119] with nitrite having a greater effect under hypoxic conditions. However, nitrite induced vasodilatation occurs across the physiological oxygen tension range.

1.7. Detection of S-nitrosothiols and other NO-carrying species

Several methods have been established for the measurement of S-nitrosothiols. There is debate as to the merits and pitfalls of each method with artefactual signal generation, decomposition or generation of S-nitrosothiol in sample processing and interference from other biomolecules in signal generation the major bones of contention.

1.7.1. Saville reaction

The classical method for S-nitrosothiol detection is the Saville reaction[120] which utilises Hg^{2+}-mediated cleavage of the S-nitrosothiol bond to liberate NO^+ from the thiol moiety. The NO^+ is then spontaneously oxidised to nitrite which can be detected by the Griess colourimetric reaction and compared to nitrite levels prior to Hg^{2+} decomposition. The Saville reaction was originally designed for non-biological *in vitro* measurement of S-nitrosothiols and problems with the method have been encountered when analysing biological S-nitrosothiols. Proteins and free reduced thiols have been shown to interfere with the Greiss reaction[121]. Furthermore, the detection limit of the Griess reaction is in the order of low micromolar concentrations of nitrite and as such cannot detect low (nanomolar) concentrations of S-nitrosothiols.

1.7.2. Chemiluminescent S-nitrosothiol detection

Chemiluminescent S-nitrosothiol detection methods rely upon releasing NO from the S-nitrosothiol in an oxygen free environment and the subsequent detection of the light emitted by the chemiluminescent reaction of NO with ozone (O_3). See Equation 8.

$$NO + O_3 \rightarrow NO_2 + O_2 + hv$$

Equation 8. Chemiluminescent reaction of NO and ozone

Chemiluminescent detection of NO by reaction with ozone is a widely validated technique originally established for the automotive industry and can also be used for the detection of nitrite and nitrate concentration by their prior reduction to NO using H^+/I^- or $H^+/I^-/Vn^{2+}$ respectively. Using this technique, it is possible to measure around 1pmole of NO in liquid[122]. All methods for the chemiluminescent determination of S-nitrosothiol concentration consist of a reaction chamber connected in line to a chemiluminescent NO analyser. However, different methods of NO release from the S-nitrosothiol in the reaction chamber have resulted in very different S-nitrosothiol concentrations being reported.

1.7.2.1. Photolytic cleavage of the S-nitrosothiol bond

The S-nitrosothiol bond is known to be sensitive to ultraviolet (UV) light[123], releasing thiyl radical (RS·) and ·NO in the process[124]. Stamler *et.al.* have developed a method of S-nitrosothiol detection based on this principle[125]. A photolysis chamber containing a 200W mercury vapour lamp was set up with a coiled borosilicate glass column running through which in turn was connected to the chemiluminescent detector via a series of cold traps, see Figure 3. The authors find a linear increase in signal recorded from the NO analyser following injection of S-nitrosothiol standards. Turning off the lamp resulted in no signal being generated. Equally, Hg^{2+} ($HgCl_2$) pre-treatment of samples resulted in >99% loss of the chemiluminescence signal. When plasma samples were obtained from healthy human

volunteers, it was found that the plasma levels of S-nitrosothiols were 7±5μM and that 90% of this signal was abolished after $HgCl_2$ incubation. Switching off the lamp again abolished the chemiluminescence signal.

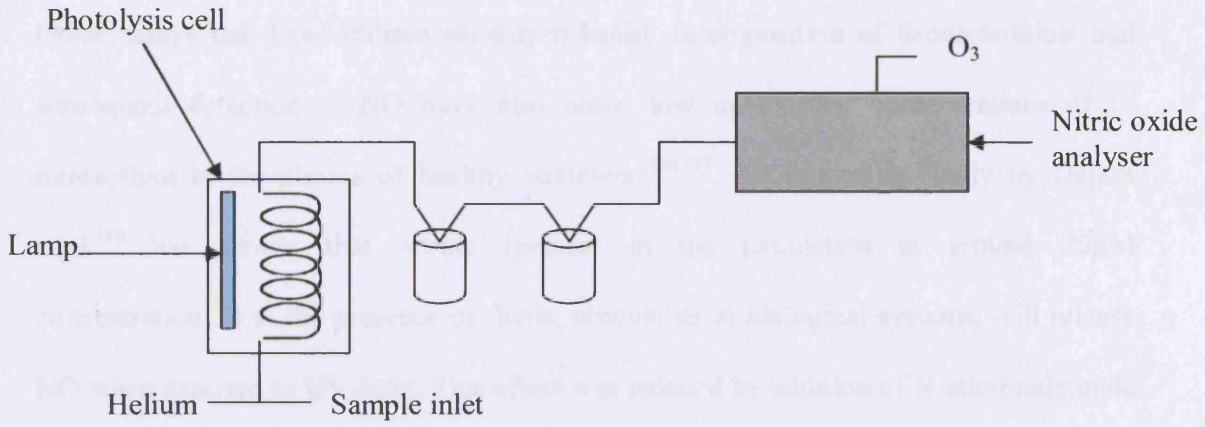


Figure 3. Apparatus for photolytic cleavage of the S-nitrosothiol bond and subsequent detection of the stoiciometrically liberated NO. Sample is injected into the sample inlet and passes through a glass coil under a stream of helium. The sample is subject to intense UV light from the lamp in the photolysis cell which homolytically cleaves the S-nitrosothiol into ·NO and RS· radicals. The released ·NO then passes through traps and is detected by chemiluminescent reaction with ozone in a nitric oxide analyser.

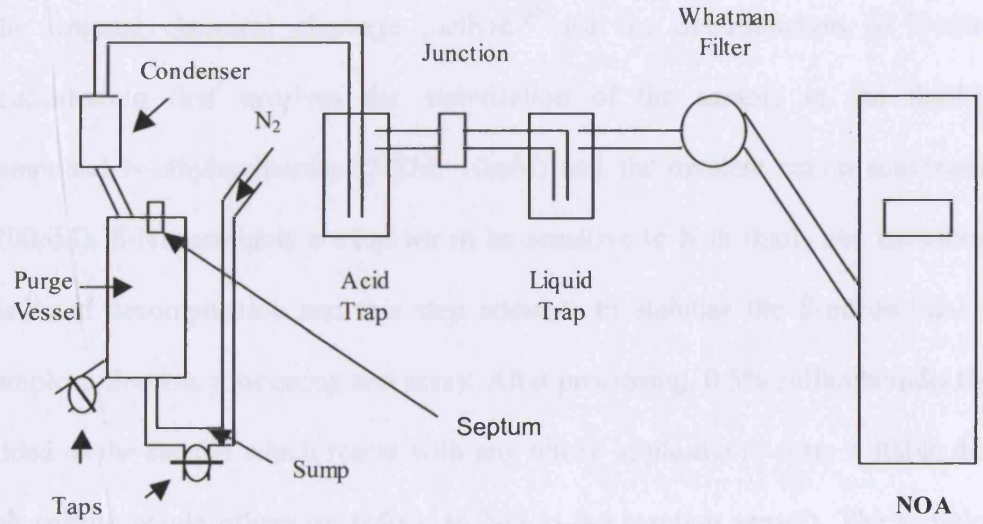


Figure 4. Apparatus for determination of S-nitrosothiol concentration by chemical cleavage method. Sample is injected into a purge vessel containing refluxing acid/KI. This cleaves the RS-NO bond liberating stoiciometric ·NO which then passes through traps into the nitric oxide analyser (NOA) where it is detected by chemiluminescent reaction with ozone.

Other assays that have utilised photolysis-based decomposition of S-nitrosothiols and subsequent detection of NO have also noted low micromolar concentrations of S-nitrosothiol in the plasma of healthy volunteers[126,127]. An interesting study by Dejam et.al.[128] has shown that nitrate (present in the circulation at around 25μM concentration[129]) in the presence of thiols, ubiquitous in biological systems, will release NO when exposed to UV-light. This effect was reduced by addition of N-ethylmaleimide (NEM, a thiol-blocking agent). This seems to suggest that photolytic-based S-nitrosothiol detection assays may well overestimate the true S-nitrosothiol concentration.

1.7.2.2. Chemical cleavage of the S-nitrosothiol bond

The original chemical cleavage method[130] for the determination of S-nitrosothiol concentration first involves the stabilisation of the sample in the thiol-blocking compound N-ethylmaleimide (NEM, 10mM) and the divalent cation scavenger DTPA (100μM). S-Nitrosothiols are known to be sensitive to both thiol- and transition metal-mediated decomposition and this step attempts to stabilise the S-nitrosothiol between sample collection, processing and assay. After processing, 0.5% sulfanilamide/1M HCl is added to the sample which reacts with any nitrite in plasma to form a stable diazonium salt (nitrite would otherwise reduce to NO in the reaction vessel). The sample is then injected into a reaction vessel containing a solution of KI, glacial acetic acid and $CuSO_4$ that is connected in line via an acid and liquid trap to the chemiluminescent detector, see Figure 4 and Figure 5.

The exact mechanism of cleavage of the S-nitrosothiol bond has not been identified, exactly but the authors suggest that this occurs through the reaction mechanism outlined in Equation 9.

$$I_2 + I^- \rightarrow I_3^-$$

$$I_3^- + 2RSNO \rightarrow 3I^- + RS^{\cdot} + 2NO^+$$

$$2NO^+ + 2I^- \rightarrow 2NO + I_2$$

$$RSNO \rightarrow RS^{\cdot} + NO$$

$$2RS^{\cdot} \xrightarrow{Cu^+} RSSR$$

Equation 9. Liberation of NO gas from S-nitrosothiols by the chemical cleavage method

When nitrite standards are compared to S-nitrosoalbumin standards (calibrated by the Saville reaction) it was found that 100pmol of S-nitrosoalbumin generated 98.9% of the signal generated from 100pmol of nitrite (without sulphanilamide). Linear standard curves for S-nitrosoalbumin were also obtained. Furthermore, spiking plasma samples with S-nitrosoalbumin resulted in stoichiometric recovery of NO per given amount of NO added, demonstrating that the assay is valid for use in biological fluids.

The plasma concentration of S-nitrosothiol detected in healthy human subjects using this method, however, is 100-fold lower than that measured by the photolytic cleavage

method. Marley et.al.[130] detect 28±7nM S-nitrosothiol in the plasma of healthy volunteers whilst Rassaf *et.al.*[131] report 30-40nM plasma concentration.

A problem with the original chemical cleavage method for S-nitrosothiol detection arose when it was discovered that incubation of biological samples with $HgCl_2$ prior to assay could not decompose all the signal generated in the assay. $HgCl_2$ (Hg^{2+}) potently releases NO^+ from the S-nitrosothiol bond, a reaction so well characterised that it is the basis for the classic Saville reaction for detection of S-nitrosothiols (see above)[120]. The method for plasma S-nitrosothiol concentration was therefore modified by Yang et.al.[132] to incorporate a $HgCl_2$ incubation step. Plasma was still collected on NEM/DTPA as before, but then subjected to various treatments before multiple sample injections to determine S-nitrosothiol, nitrite and the Hg-stable fraction.

As described in figure 4, after collection and centrifugation to obtain plasma, 3 tubes are set up with equal volumes of plasma added. One plasma sample is injected directly into the reducing solution in the purge vessel (tube 3). Sulphanilamide is then added to tube 1; this is injected and the signal recorded. Finally, the plasma in tube 2 is exposed to $HgCl_2$ prior to injection and then injected into the purge vessel. The signal recorded from tube 3 therefore corresponds to nitrite + S-nitrosothiol + Hg-stable species, from tube 1, S-nitrosothiol + Hg-stable species and tube 3 Hg-stable species alone. Therefore, by subtraction of the signals the concentration of each species can be elucidated.

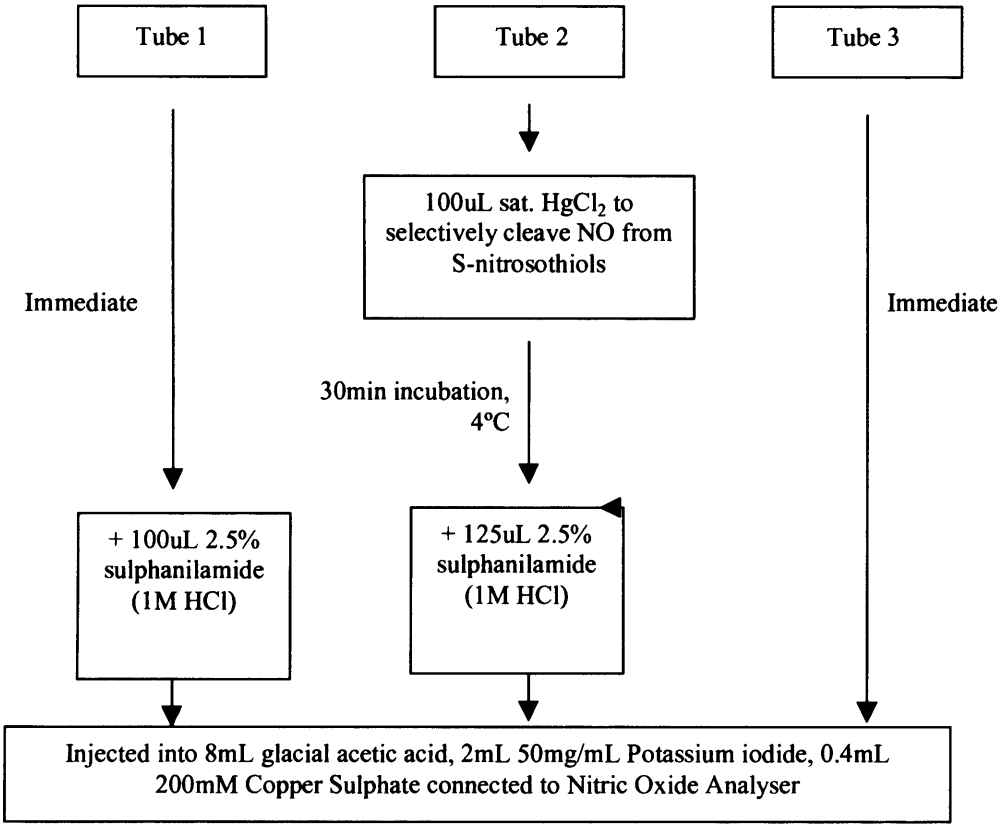


Figure 5. Modified method for determination of plasma S-nitrosothiol, nitrite and Hg-stable species concentration

The identity of the Hg-stable species has not been fully established. The current hypothesis, proposed by Feelisch et.al. is that, considering the stability of the species and the liberation of NO from it under reducing conditions, is that it constitutes an N-nitrosamine of some description[133].

1.8. Cirrhosis

1.8.1. Outline and causes of cirrhosis

Cirrhosis occurs following chronic liver injury due to alcohol abuse, chronic viral hepatitis, chronic cholestasis, iron overload or autoimmune disease. It is characterised by disruption of the normal liver architecture by fibrosis and regenerative nodule formation. Once liver disease has progressed to cirrhosis, the damage observed is irreversible and the prognosis is poor if the insult is not withdrawn. The liver is the largest internal organ and is a centre of metabolic activity being a major site of energy metabolism, substrate interconversion, protein synthesis, storage and toxin/waste product detoxification and clearance. Furthermore, the liver receives about 25% of cardiac output and the development of hepatic fibrosis, nodules and altered sinusoidal resistance in cirrhosis leads to increased vascular resistance within the liver and elevation of portal pressure. The combination of decreased liver cell function and portal hypertension can lead to several complications including impaired haemostasis, hepatic encephalopathy, renal dysfunction and ascites.

1.8.2. Bile duct ligation model of cirrhosis in the rat

Chronic biliary obstruction (3-4 weeks in the rat) leads to the development of a biliary cirrhosis with the formation of ascites, splenomegaly and hyperbilirubinaemia[134].

Histological abnormalities include proliferation of bile ducts, cell necrosis and bridging fibrosis with features of biliary cirrhosis[135]. The chronic bile duct ligation (BDL) model of cirrhosis is also associated with the development of a haemodynamic dysfunction[136] see Table 1.

Haemodynamic parameter	Normal	Cirrhotic
Cardiac Index (CI)	16±1ml/min	30±2ml/min
Mean Arterial Pressure (MAP)	114±3mm Hg	104±4mm Hg
Portal Pressure (PP)	9±0.5mm Hg	15±0.5mm Hg
Systemic vascular resistance (SVR)	7±0.5mm Hg/mL	4±0.5mm Hg/mL

Table 1. Haemodynamic parameters in the BDL model of cirrhosis. Figure re-drawn from reference 136.

Renal function in BDL cirrhotic rats is also impaired (lower GFR) as measured by decreased sodium excretion[137] and decreased creatinine clearance[138].

1.8.1. Complications of cirrhosis

1.8.1. Sepsis-like syndrome in cirrhosis

Bacterial infection is a common complication of decompensated cirrhosis. The risk of bacterial infection in cirrhosis increases with the severity of the disease progression; the infection rate in hospitalised cirrhotic patients has been determined to be 32-34%[139,140]. However, the same studies show that, if patients are admitted with gastrointestinal bleeding this increases, the risk of infection to 78%. This compares to a general hospital population rate of infection of 5-7%[141]. Infection in cirrhosis is linked to increased mortality over non-infected cirrhotic patients[140] and is mainly manifested as spontaneous bacterial peritonitis (SBP) in ascitic fluid, though urinary tract infections and pneumonia are also observed.

1.8.2. Cytokine induction in cirrhosis

Cytokines are signalling peptides produced by cells under physiological and pathological conditions. They are particularly, but not exclusively, associated with the immune response. In liver disease there is increased synthesis of a variety of cytokines, notably tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in response to endotoxin or local tissue injury[142]. TNF- α is essential for normal liver regeneration as demonstrated by the observation that anti-TNF-α antibodies inhibit liver regeneration[143]. IL-6 also appears to be necessary for liver regeneration but also requires TNF-α for induction[144].

TNF-α and IL-6 both induce transcription factors to modulate their effects. TNF-α activates nuclear factor-κB (NFκB) whilst IL-6 activates signal transducer and activator of transcription 3/5 (STAT3/5)[145].

However, TNF-α has also been shown to be involved in the development of many experimental liver injury models including D-galactosamine/endotoxaemia[146] and carbon tetrachloride intoxication[147]. TNF-α has also been implicated in extracellular protein synthesis in cirrhosis[148] and in the development of the hyperdynamic circulation in portal hypertensive rats[149]. Infusion of anti-TNF-α antibodies to portal hypertensive rats resulted in amelioration of the hyperdynamic circulation but had no effect on control rats.

1.8.4. Impaired haemostasis in liver disease

Haemostatic abnormalities are commonly observed in liver disease. The liver is the major site of catabolism and metabolism of most of the clotting and anticoagulant factors involved in the coagulation cascade. The balance of these factors is critical in the maintenance of normal haemostasis. There are also platelet abnormalities in liver disease, both in their production, splenic sequestration and activity.

1.8.4.1. Bacterial infection and haemostasis in liver disease

Although there has been much research on platelet aggregation in the context of liver disease, the presence or absence of infection has never been assessed despite observations that bacterial infection is a risk factor for variceal bleeding[150,151,152].

1.8.4.2. Coagulation factor abnormalities in liver disease

Coagulation factor number	Alternative name
Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Tissue thromboplastin
Factor IV	Calcium
Factor V	Proaccelerin
Factor VI	(Factor Va)
Factor VII	Proconvertin
Factor VIII	Antihemophilic factor
Factor IX	Christmas factor
Factor X	Stuart-Prower factor
Factor XI	Plasma thromboplastin antecedent
Factor XII	Hageman factor
Factor XIII	Fibrin-stabilising factor

Table 2. List of coagulation factors

The majority of the coagulation factors are synthesised in the liver and as such their concentrations are usually decreased in patients with hepatic failure[153,154]. However, factor XIII levels are increased. Various explanations for this anomaly have been proposed including a possibly increased synthesis of factor VIII from other tissues (factor VIII expression has been found in a variety of tissues[155], though in physiological conditions liver synthesis is considered to predominate as liver transplant has been shown to resolve haemophilia[156]). Alternatively, decreased liver function may result in decreased clearance of factor VIII or the liver may continue to synthesise factor VIII from sinusoidal endothelial cells despite a reduction in hepatocyte synthesis[155].

The γ-carboxylation of glutamic acid residues in factor II, VII, IX and X, protein C, protein S and protein Z is a vitamin K dependant process which facilitates their binding to anionic phospholipids[157,158]. Dysfunctional post-translational modification of coagulation factor proteins and an associated decrease in their ability to migrate to the site of thrombosis may play a role in impaired haemostasis in liver disease.

Synthesis of proteins C, S and Z as well as antithrombin is decreased in liver disease[154]. The end result is decreased functional and altered levels of anti-coagulant factors leading to impaired coagulation in cirrhosis.

1.8.4.4. Platelet dysfunction in liver disease

Platelet production, sequestration and aggregation are abnormal in cirrhosis. Thrombocytopenia, due to a combination of decreased platelet formation secondary to decreased synthesis of thrombopoetin in the liver, and increased sequestration of platelets by an enlarged spleen.

1.8.4.4.1. Platelet aggregation dysfunction in liver disease

Impaired platelet aggregation in liver disease is widely recognised and was first described by Thomas *et.al.* in 1967[159]. Impaired platelet aggregation is independent of thrombocytopaenia[162] and corresponds to the severity of liver disease[160]. Impaired platelet aggregation in cirrhosis is associated with decreased intraplatelet Ca^{2+}

concentration[161] and IP_3 concentration[163], decreased synthesis of thromboxane A_2[162] and increased intraplatelet concentration of cGMP and cAMP[163].

1.8.4.4.2. Intrinsic or humoral dysfunction of platelet aggregation

At present there is some controversy as to whether inhibition of platelet aggregation in cirrhosis is intrinsic to the platelet, or whether it involves a circulating humoral inhibitor of platelet aggregation. In support of an intrinsic abnormality, washed platelets from cirrhotic patients have decreased aggregation compared to healthy volunteers[162,164]. However, recent studies have shown that there may also be a humoral component to the platelet dysfunction observed in cirrhosis.

TxA_2 and the prostaglandins are metabolites of arachidonic acid (AA). The majority of AA is synthesised in the liver from 18-carbon precursors (predominantly dietary linoleic acid) and liver disease leads to decreased levels of AA in cellular membranes. Linoleic acid supplementation had no effect on platelet aggregation in cirrhotic patients[165] but supplementation of AA caused a significant increase in platelet aggregation (42±3% with placebo and 56±3.5%)[166]. An increase in plasma AA (74%) and a small increase in RBC AA composition (7%) accompanied this increase in platelet aggregation, but platelet AA could not be measured due to thrombocytopaenia.

Altered lipoproteins in liver disease have also been shown to have an effect on platelet aggregation. Early studies into the effect of high-density lipoprotein (HDL) on platelet

aggregation reported conflicting results, either an inhibitory effect was noted[167] or no effect[168]. When HDL subclasses were separated and analysed for anti-platelet activity it was found that apolipoprotein E (apoE), a 34 kDa polypeptide from the HDL_2 fraction, was a potent inhibitor of platelet aggregation[169,170]. A correlation has since been demonstrated between abnormally apoE-rich HDL in cirrhotic patients and impaired platelet aggregation[171]. The mechanism of the apoE effect on platelet aggregation has been investigated and has been shown to be mediated through the L-arginine-NO signal transduction pathway. L-[^{3}H]arginine was converted to L-[^{3}H]citrulline in a 4 times greater concentration when apoE was exposed to platelets than in control platelets[172]. It was also found that NOS inhibitors prevented 75% of ApoE mediated inhibition of platelet aggregation indicating ApoE inhibits platelet aggregation primarily through a cGMP dependent mechanism.

The report of Laffi et.al. demonstrates that, whilst cAMP and cGMP concentration are increased in cirrhotic platelets compared to those from healthy volunteers, cirrhotic and healthy platelets produce identical amounts of cAMP and cGMP in response to PGI_2 and nitric oxide (NO)[163]. cAMP and cGMP accumulate in response to PGI_2 and NO respectively and are the known intraplatelet inhibitors of platelet aggregation. Both PGI_2 and NO synthesis are known to be increased in cirrhosis[173,174,175] and are formed in the vascular endothelium. A correlation has also been made between the observation that PGI_2 concentration in portal blood samples is higher than in samples from the systemic circulation in cirrhotic patients[176] and the observation that platelet aggregation is more impaired in platelets isolated from cirrhotic portal blood than from systemic blood[177].

A complete plasma crossover study has been carried out in platelets and plasma from cirrhotic patients and healthy volunteers[178]. Intriguingly, the addition of cirrhotic plasma to healthy washed platelets resulted in increased platelet aggregation compared to when healthy washed platelets were combined with healthy plasma. The authors speculate that this observation may indicate an upregulation of a humoral factor which activates platelet aggregation in cirrhosis as a compensatory mechanism for decreased intrinsic platelet aggregation. However, the authors also point out that their method for platelet washing has not been validated for aggregation studies and was designed for measurement of intraplatelet Ca^{2+} concentration. It must also be noted that the cirrhotic platelets in this experiment do not aggregate less than those of healthy controls and that the standard deviation in each experiment are very large.

1.8.5 Vascular dysfunction in liver disease and hepatorenal syndrome

1.8.5.1. Portal hypertension, lymph formation and ascites formation

Portal hypertension in cirrhosis is due to a combination of increased resistance to blood flow through the liver and increased portal blood flow secondary to splanchnic vasodilatation. The increased forward flow of blood into the splanchnic capillaries leads to increased splanchnic capillary hydrostatic pressure and increased extravasation of a protein rich fluid[179] with increased capillarisation of the hepatic sinusoids in cirrhosis[180]. The resulting collagen deposition in the space of Disse causes blockage of the large

hepatic fenestrae reducing sinusoidal protein permeability and therefore hepatic lymph protein concentration[181]. The combination of these two processes in cirrhosis results in increased lymph fluid formation exceeds the ability of the lymphatic system to drain the fluid. The result of this is formation of fluid in the abdominal cavity termed ascites.

1.8.5.2. Sodium retention and ascites formation

Sodium retention in cirrhosis leads to the formation of ascites. The retention of sodium is secondary to increased renal tubular reabsorption[182], partly mediated by activation of the renin-angiotensin-aldosterone-system (RAAS)[183] and sympathetic nervous system[184] in response to decreased mean arterial pressure (as a result of vasodilatation). The reduction of arterial pressure leads to renal vasoconstriction and ultimately the development of the hepatorenal syndrome.

1.8.5.3. Nitric oxide and splanchnic vasodilatation

Advanced cirrhosis is associated with a hyperdynamic circulation in which cardiac output is elevated and vascular resistance is decreased[185]. The observation that the hyperdynamic circulation is associated with vasodilatation and systemic endotoxaemia led to speculation that endotoxaemia may cause induction of iNOS leading to vasodilatation[186]. NO synthesis is increased in animal models of cirrhosis as well as patients with cirrhosis.

Thus plasma nitrite/nitrate concentration is higher in cirrhotic rats[187,188] and humans[189] and aortic cGMP is elevated in cirrhotic rats[190].

1.8.5.3.1. In vivo administration of NOS inhibitors

Administration of a NOS inhibitor leads to increased blood pressure in normal and cirrhotic rats[191] and also normalisation of aortic cGMP levels and reduced plasma renin activity and vasopressin concentration in cirrhotic rats[192]. Further, renal function was improved in cirrhotic rats following NOS inhibitor infusion[193,194,195].

Infusion of NOS inhibitors to humans leads to a decrease in forearm blood flow in decompensated cirrhotic patients[196]. However, compensated cirrhotic patients show no change[197] or a smaller decrease[196] in forearm blood flow following NOS inhibitor infusion. However, whilst the blood pressure and SVR increased and heart rate and cardiac output decreased upon NOS inhibitor infusion in cirrhotic patients, renal function is not improved, probably due to decreased renal blood flow[198,199].

One problem with the use of NOS inhibitors in cirrhosis is that they enhance liver injury in several models of disease as assessed by transaminase levels their[200]. Infusion of L-NAME also exacerbated renal dysfunction in cirrhotic rats[201].

1.8.5.3.2. In vitro analysis of isolated vascular tissue

Isolated vascular tissue from cirrhotic rats has provided insight into the role of NO in the hyperdynamic circulation. Isolated rings of mesenteric artery and thoracic aorta from portal hypertensive and CCl_4 cirrhotic rats show hypo-responsiveness to the pressor effects of nor-adrenaline, potassium and arginine-vasopressin compared to control rats[202,203,204]. Incubation with a NOS inhibitor could partially, but not completely, restore the pressor effect in these vessels. CCl_4 cirrhotic rats also have increased sensitivity to ACh, but not to an endothelium-independent vasodilator, an effect that was again partially abolished by incubation with a NOS inhibitor[205].

BDL cirrhotic rats also have impaired vasoconstrictor responses compared to controls[206,207,208]. However, there was no change in the response to vasodilators in the BDL model of cirrhosis. NOS inhibition in BDL cirrhotic rats resulted in complete restoration of pressor response[206] rather than the partial restoration observed in CCl_4 cirrhotic rats.

1.8.5.4. Plasma nitric oxide and S-nitrosothiol concentration in cirrhosis

1.8.5.4.1. Nitric oxide

Plasma nitrite/nitrate (NOx) concentration has consistently been shown to be elevated both in patients and in rat models of cirrhosis[209,210]. This pattern of increased plasma NOx

concentration was confirmed in BDL cirrhotic rat plasma, however, different labs have reported different quantities, possibly due to different measurement techniques or rat sources. Whilst Yang et.al. report plasma NOx concentration increase from 7±0.1μM in normal rats to 23±1μM in BDL rats[211], Vazquez-Gil et.al. report an increase from 41.1±2.9μM in normal rats to 53.3±5.9μM, 16 days post-bile duct ligation and 74.7±8.1μM 31 days post-bile duct ligation[212]. Marley et.al. report 29.4±2.2μM and 40.3±2.7μM in normal and BDL rats respectively[229]. A similar increase in plasma NOx is seen in CCl_4 cirrhotic rats in which a portal-arterial NOx gradient was also observed[213]. Arterial and portal NOx concentration in normal rats were 29.1±6.1μM and 24.7±4.7 respectively. Following induction of cirrhosis arterial NOx concentration increased to 93.1±22.4μM and portal NOx concentration increased to 127.1±27.2μM.

Guarner et.al. have correlated NOx levels to endotoxaemia in patients with cirrhosis[214]. This correlation has been demonstrated experimentally in BDL cirrhotic rats[215]. Injection of 0.5mg/Kg LPS to BDL cirrhotic rats time dependently increased plasma NOx concentration. See Table 3.

Time	Normal rats	BDL cirrhotic rats
0	29±4μM	82±19μM
1	50±11μM	81±7μM
3	84±17μM	167±19μM
6	172±23μM	179±39μM

Table 3. Effect of endotoxin on nitric oxide synthesis in normal and cirrhotic rats. Table re-drawn from reference 215.

Decompensated cirrhosis leads to increased circulating endotoxin. Thus, one might expect plasma NOx to be higher in patients with decompensated liver disease as opposed to compensated cirrhosis. Thus, Lluch *et.al.* observe similar levels of plasma NOx in healthy controls and compensated cirrhotic patients (40 ±5μM and 37±3μM), decompensated patients had highly elevated plasma NOx (97±10μM)[216].

Thus NO synthesised in cirrhosis increased in patients with severe liver disease, possibly secondary to endotoxaemia. There is also increased NO synthesis in the splanchnic vascular bed compared to the systemic circulation, which may explain splanchnic vasodilatation in the hyperdynamic circulation.

1.8.5.4.2. S-Nitrosothiols

S-Nitrosothiol concentrations are also increased in the BDL rat model of cirrhosis[217]. Plasma S-nitrosothiol concentration increases from 51±6nM in normal rats to 206±59nM in BDL cirrhotic rats. In correlation with increased NO production in cirrhosis following endotoxaemia (see above), S-nitrosothiol concentration rises markedly following injection of endotoxin. Plasma S-nitrosothiol concentration rose from 206±59μM in BDL cirrhotics to 1335±423nM following injection of 0.5mg/Kg LPS, 2hours. However, the same dose of LPS to normal rats only caused an increase from 51±6nM to 108±23nM. This suggests that cirrhotic rats are hypersensitive to endotoxin, possibly due to the synergistic elevation of cytokines and/or NO production induced by cirrhosis and endotoxin. This is supported by the observation that plasma TNF-α concentrations, whilst

increasing to a similar extent in cirrhotic rats tand normal rats, is sustained for a prolonged period of time in BDL cirrhotic rats following endotoxin challenge than in normal rats (See Figure 6)[215]. Basal TNF-α concentration in normal and BDL rats was found to be 9±1pg/mL and 52±22pg/mL respectively. Injection of 0.5mg/Kg LPS resulted in a dramatic increase in plasma TNF-α concentration in both normal and BDL cirrhotic rats after 1hour, however, the concentration fell quickly in normal rats but not in cirrhotic rats. See Figure 6.

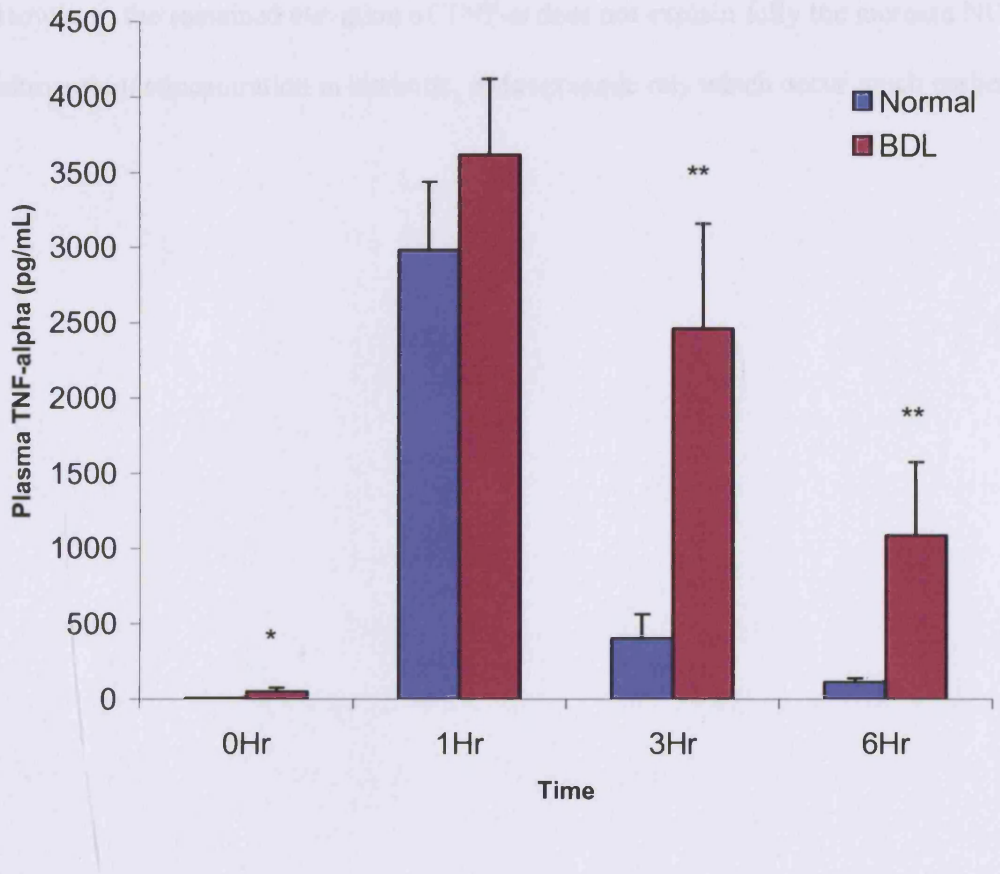


Figure 6. Plasma TNF-α concentration in normal and cirrhotic rats following injection with 0.5mg/Kg LPS. Whilst a similar initial rise in TNF-α concentration is observed in both normal, non-cirrhotic rats and in cirrhotic rats following injection of LPS the level is elevated longer and at a higher level in cirrhotic rats than in the non-cirrhotic rats. This suggests cirrhotic rats are more sensitive to endotoxin than are normal rats. Figure re-drawn from reference 215.

Interestingly, however, NFκB activation was measured in the same study and although the basal activation was 5-fold higher in cirrhotic rats than normal rats, the elevation seen after LPS challenge was no different. The sustained elevation of TNF-α in cirrhotic rats may therefore be either the result of increased formation or decreased clearance. However, the sustained elevation of TNF-α does not explain fully the increase NO and S-nitrosothiol concentration in cirrhotic, endotoxaemic rats which occur much earlier.

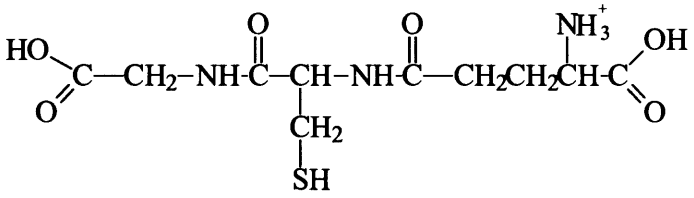
1.8.6. Thiol redox status and cirrhosis

Thiols in physiological systems can be broadly split into two categories: high molecular weight (HMW) protein thiols and low molecular weight (LMW) non-protein thiols. Thiol redox status is extremely important in physiology. Thiol groups exist either as free sulphydryls (R-SH) or as disulphides (RS-SR). The disulphides formed can be mixed disulphides (LMW-HMW, LMW_1-LMW_2) or common species disulphides e.g. cystine (cysteine-cysteine).

The major plasma protein thiol is albumin, which is present in the circulation at 40g/L concentration and has one vicinal cysteine residue (Cys34) per protein. Low molecular weight thiols in the rat circulation comprise of predominantly cysteine (13±0.4μM cysteine and 90±5μM disulphide)[218], glutathione (15-20μM GSH and 1-1.5μM disulphide)[218] and homocysteine (HSCH); (1μM HCSH and 4μM disulphide)[219]. Glutathione in the plasma is predominantly synthesised in and exported from the liver[220].

1.8.6.1. Thiol antioxidants

1.8.6.1.1. Glutathione antioxidant system



Glycine Cysteine Glutamine

Figure 7. Structure of Glutathione

The glutathione antioxidant system is the most abundant antioxidant system in mammals. It relies upon the oxidation of two reduced glutathione molecules (2GSH) to form the disulphide (GSSG) with concomitant reduction of the oxidant species. See Equation 10.

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

Equation 10. Glutathione mediated reduction of hydrogen peroxide

Oxidised GSSG can then be reduced back to two GSH molecules by the enzyme glutathione reductase (GR) and its essential cofactor NADPH. The ratio of GSH:GSSG therefore provides an indication of oxidative stress. Intracellular concentration of GSH is very high, up to 20mM in liver; GSH:GSSG ratio is maintained at around 200:1 in normal tissue[220]. Glutathione is by far the most abundant intracellular thiol.

1.8.6.1.2. N-acetylcysteine

N-acetylcysteine (NAC) has the structure shown below

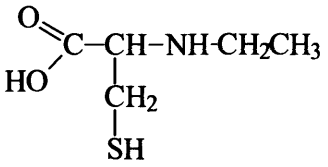


Figure 8. N-acetylcysteine structure

The rate-limiting step in the synthesis of glutathione is the availability of cysteine. NAC can be taken up by cells and deacetylated to cysteine and leads to increase of glutathione synthesis[221]. NAC is used clinically in the treatment of paracetamol overdose. The hepatotoxic paracetamol metabolite, N-acetyl-ρ-benzoquinone (NAPQI) is detoxified by GSH which, following a large paracetamol overdose, can be completely consumed. NAC infusion increases hepatic GSH levels and the ability of the liver to detoxify NAPQI[222]

Administration of NAC to rats and humans with liver disease has been shown to be improve cardiovascular function and renal function. Administration of NAC to acute cholestatic rats resulted in increased glomerular filtration rate (GFR)[223]. NAC administration to portal hypertensive rats prior to induction of hypertension resulted in the prevention of the hyperdynamic circulation[224]: Cardiac index and portal pressure were not elevated as they were in the untreated group and systemic vascular resistance (SVR) was normalised. Further NAC administration to patients with hepatorenal syndrome significantly improved GFR and survival[225].

Low molecular weight thiols have, as mentioned previously, been shown to increase the decomposition rate of S-nitrosothiols *in vitro*. This effect has recently been shown *in vivo* by Orie et.al. whereby co-infusion of S-nitrosoalbumin and NAC to rats results in increased decomposition of S-nitrosothiols compared to S-nitrosoalbumin infusion alone. This was accompanied by a decrease in aortic and renal blood flow following NAC co-infusion. This observation, along with elevation of S-nitrosothiol concentration in liver disease may explain the fall in blood pressure in patients with acute or chronic liver disease, but not healthy controls[226,227].

1.8.6.1.3. Lipoic acid

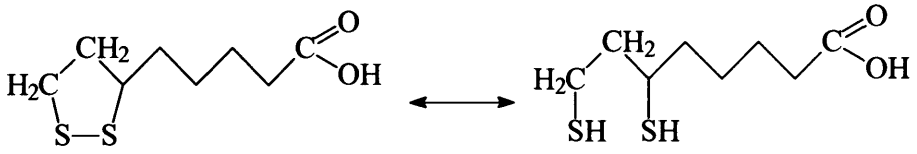


Figure 9. Lipoic acid (left) and dihydrolipoic acid (right)

Lipoic acid (LA, otherwise known as thioctic acid) exists in either the oxidised LA form or as its reduction product dihydrolipoic acid (DHLA). Lipoic acid has both antioxidant and transition metal chelator properties[228]. Lipoic acid has been shown to improve haemodynamic and renal dysfunction in liver disease. Renal function in acute cholestatic rats was improved slightly by intraperitoneal LA administration though not as much as with NAC administration[223]. However, LA was extremely effective in lowering

isoprostane formation in this model. This observation confounds the hypothesis that the accumulation of isoprostanes in liver disease contributes to renal dysfunction through their vasoconstrictor activity.

LA has also been demonstrated to prevent the hyperdynamic circulation in cirrhotic rats[229]. Lipoic acid administration in the drinking water of cirrhotic rats prior to induction of cirrhosis resulted in normalisation of cardiac index (CI) and systemic vascular resistance (SVR) compared to untreated cirrhotics in which CI was elevated and SVRI decreased. Portal pressure, elevated in untreated cirrhotics (47% higher than controls), was decreased 16% following LA administration.

1.8.6.2. Thiol antioxidants, cytokines and transcription factors in cirrhosis

Thiol redox state has been shown to mediate transcription factor activation. NFκB is a transcription factor associated with inflammatory cytokines, notably TNF-α. TNF-α is upregulated in cirrhosis and is associated with the development of the hyperdynamic circulation. TNF-α inhibitors ameliorate the hyperdynamic circulation and lower nitric oxide synthesis in cirrhotic rats[230]. NFκB, as mentioned before is a critical transcription factor in iNOS transcription. NAC has been shown to elevate intracellular glutathione concentration and inhibit NFκB activation and nuclear translocation in a human T-cell line[231], an effect also observed with lipoic acid[232]. However, lipoic acid has also been shown to inhibit NFκB activation in glutathione synthesis arrested cells[233]. It is therefore speculated that thiol mediated disruption of TNF-α and NFκB-mediated gene induction

may be critical to the ameliorative effect of thiol antioxidants to the hyperdynamic circulation in liver disease[224].

1.9. Analbuminaemic rat strain

1.9.1. Origin

The analbuminaemic strain of rats was characterised by Nagase et.al. in 1979[234]. They were, however, first recognised by Nagase in 1974 in dead hypercholesterolaemic Sprague Dawley rats but no live animals could be found in the colony. The analbuminaemic strain described in 1979 is derived from interbreeding a hypercholesterolaemic Sprague Dawley rat strain established by Hattori et.al.[235]. These hypercholesterolaemic rats were established by interbreeding Sprague Dawley rats that were high responders to high cholesterol diet. It was found in the breeding experiments that analbuminaemia is inherited as an autosomal recessive trait[234].

1.9.2. Characterisation of the analbuminaemic rat strain

The original experiments of Nagase *et.al.*[234] showed the analbuminaemic (NAR) strain to be characterised by extraordinarily low serum albumin content and a hyperlipidaemia. Total serum protein concentration was, however, the same in normal Sprague Dawley and NAR strains due to and increased synthesis of globulin. The relative concentration of

albumin and proteins in normal male Sprague Dawley and NAR strains is shown in Table 4. No parameter was significantly different in female rats of either strain.

Protein	Normal Sprague Dawley	Analbuminaemic (NAR)
Total protein (g/dL)	6.6±0.8	6.5±1
α_1-globulin	16±5%	35±4%
α_2- and α_3-globulin	9±2%	12.5±1%
β-globulin	16±1%	28±3%
γ-globulin	13±6%	21±6%
Albumin	46±8%	3.5±1%

Table 4. Total protein concentration and relative distribution of albumin and globulin in serum of normal Sprague Dawley and NAR rats. Table re-drawn from reference 234.

There is also no albumin expression in the tissue of NAR rats[236]. No albumin could be found in the skin, muscle, small intestine, kidney or liver of NAR rats. The explanation for the mechanism of albumin deficiency in the NAR strain was elucidated by analysis of liver albumin mRNA. It was found that there is a 7-base-pair deletion at the 5' end of an albumin gene intron and that this intron sequence is preserved in mRNA precursors isolated from liver nuclei of analbuminaemic rats[237]. The nucleotide sequence at the 5' end of introns is highly conserved in many eukaryotic genes and is considered to be important in mRNA splicing[238]. It therefore appears that the seven-base-pair deletion in the albumin gene of analbuminaemic rats blocks albumin mRNA splicing in the analbuminaemic rat liver and this leads to the development of analbuminaemia.

An examination of lipoproteins in 1 year-old analbuminaemic rats[239] has shown that cholesterol content of NAR rats is 6.1±0.3mM vs. 2.5±0.2mM in normal Sprague Dawley (SpD) rats. The majority of cholesterol has been shown to be located in the LDL and HDL2 density range. Whilst apolipoprotein A-I and B are elevated in NAR rats (3-4-fold and 2-fold respectively) compared to SpD rats there was no difference in the level of apolipoprotein A-IV or E between strains. However, the same authors have reported strikingly different results in 250-300g rats (~3months-old). See Table 5 and below.

Only triglyceride concentration is affected by 28-day administration of pravastatin. However, all plasma lipid and lipoprotein concentrations (except ApoA-IV) measured were significantly ($p < 0.01$) higher in NAR rats compared to normal SpD rats. Notable is the ~50% higher ApoE concentration in NAR rats than SpD rats as elevated synthesis of ApoE is known to decrease platelet aggregation in cultured cell lines[170].

	Saline		Pravastatin	
Fraction	**NAR**	**SpD**	**NAR**	**SpD**
Total cholesterol (mM)	9.9±0.3	2.8±0.1	9.1±0.7	2.7±0.1
Unesterified cholesterol (mM)	3.3±0.1	1.0±0.1	3.3±0.2	1.0±0.1
Cholesterol ester (mM)	6.6±0.2	1.8±0.0	5.7±0.6	1.7±0.0
Triglyceride (mM)	5.3±0.3	0.7±0.1	3.0±0.6	0.7±0.1
Phospholipid (mM)	7.2±0.2	2.4±0.1	6.4±0.5	2.4±0.1
ApoA-I (mg/100mL)	145±5	31±2	142±4	29±2
ApoA-IV (mg/100mL)	15.2±0.4	16.6±1.2	15.8±1.8	16.6±0.9
ApoE (mg/100mL)	31.1±1.1	19.4±0.6	31.3±2.2	20.5±4.2
ApoB (arb)	166±5	83±2	165±6	85±8

Table 5. Plasma lipid and lipoprotein concentration in analbuminaemic (NAR) and Sprague Dawley (SpD) rats with and without pravastatin. Table re-drawn from reference 239

1.9.3. Functional effect of analbuminaemia on platelet aggregation and blood pressure

Analbuminaemic (NAR) rats have previously been used to examine the role of S-nitrosoalbumin in the circulation. The blood pressure of normal Wistar and NAR rats was compared following intravenous NO-donor infusion and the concentration of S-nitrosothiol generated compared[240]. It was found that 5μmol/Kg NOC-7 (an NO-donor) infusion to both Wistar and NAR rats caused a similar 60±6.1 and 63±3.5 mm/Hg drop in blood pressure respectively. However, the recovery time to normal blood pressure was significantly different: 61.3±10.4 min vs. 35.3±6.7min in Wistar and NAR rats respectively, see Figure 10. There was no difference between the strains in the recovery times to papaverine infusion. S-Nitrosothiol concentration following NOC-7 administration was measured by the Saville assay and was shown to be 3-fold higher in normal Wistar rats than in NAR rats.

Collagen-induced platelet activation and aggregation is also altered in analbuminaemic rats[241]. NAR rats do not respond to low concentrations of collagen 1.25-2.5μg/mL unless albumin (with bound fatty acids or fat-free albumin) is supplemented. However, at high collagen concentration (5μg/mL) platelet aggregation is identical to that in normal Sprague Dawley rats. The authors correlate this dysfunction to collagen-induced synthesis of 12-hydroxyeicosatetraenoic acid (12-HETE), which is formed in response to collagen-induced aggregation, but not ADP induced aggregation. 12-HETE has been shown to inhibit platelet aggregation through interference of arachidonic acid liberation from phospholipids[242]. In the absence of albumin, 12-HETE was found to be retained in

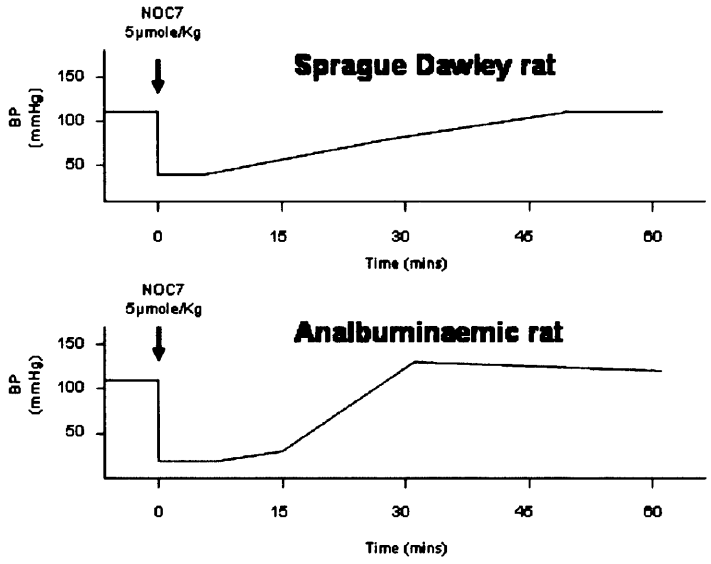


Figure 10. Typical tracing of the hemodynamic effect of NOC7 injection in normal Sprague Dawley (control) rats and analbuminaemic (NAR) rats. Triangle, 50% recovery time. Diamond, 100% recovery time. Injection of the NO donor NOC7 resulted in a dramatic fall in blood pressure in both NAR and control rats. However, the recovery time to normal blood pressure was much shorter in NAR rats. This suggests that the effect of NO in the vasculature is influenced by the presence or absence of albumin. S-nitrosoalbumin is the most abundantly formed S-nitrosothiol in the circulation and also has a much linger half life than NO. This data therefore suggests that analbuminaemic rats may recover from NOC7 challenge quicker than control rats because they don't form circulating S-nitrosoalbumin to potentiate the vasodilatory effect of NO. NAR rats may therefore also be a good model to study the effects of S-nitrosothiols in disease states in which circulating S-nitrosothiols are implicated. Figure re-drawn from Minamiyama *et.al.*[240]

The platelet and so inhibit platelet aggregation. Supplementation of albumin restored the platelet aggregation induced by low dose collagen and resulted in secretion of 12-HETE into the medium. Albumin is therefore necessary for collagen induced platelet activation and aggregation. However, in the same study no difference was found in ADP stimulated aggregation between normal Sprague Dawley and NAR rats.

Initial Aims and Hypothesis

Earlier studies from our laboratory have shown that circulating S-nitrosothiols are increased in cirrhotic rats, and are markedly increased in endotoxaemic cirrhotic rats. In other experiments it has also been shown that administration of low molecular weight thiols can ameliorate vascular dysfunction in animal models of liver disease. A further experiment has shown that S-nitrosothiol concentration is reduced in S-nitrosoalbumin infused rats following blous injection of N-acetylcysteine.

The hypothesis of this thesis is therefore that elevated circulating S-nitrosothiols may be responsible for platelet and vascular dysfunction in cirrhosis and that this dysfunction may be treated by administration of low molecular weight thiol antioxidants. Further, as S-nitrosothiols are markedly elevated in endotoxaemic cirrhotic rats, the worsening of vascular dysfunction and haemostasis observed in the infected state may also be explained by the associated increase in circulating S-nitrosothiols via NO mediated vasodilatation and inhibition of platelet aggregation.

Experiments are therefore carried out using a rat model of endotoxaemia and cirrhosis to firstly assess whether dysfunctional platelet aggregation is responsible for the increased risk of bleeding that has been associated with infection in cirrhotic patients. The potential therapeutic effect of administration of low molecular weight thiol antioxidants on platelet aggregation in cirrhosis is then investigated. Plasma S-nitrosothiol concentration is also re-assessed in these experiments as methodological advances in the tri-

iodide/chemiluminescence S-nitrosothiol assay have shown that a significant proportion of signal previously attributed to S-nitrosothiol formation has been found to be non-mercury labile. The proportion of mercury labile S-nitrosothiol and other NO carrying species is therefore re-assessed.

As the principal plasma S-nitrosothiol has been shown to be S-nitrosoalbumin, a model of cirrhosis is established in an analbuminaemic strain of rats to compare the circulating S-nitrosothiol concentration and platelet dysfunction in the absence of an albumin thiol reservoir to that in our normal strain of Sprague Dawley rats expressing normal levels of albumin.

We have previously shown amelioration of vascular dysfunction following administration of low molecular weight thiols in various models of liver disease, as assessed by haemodynamic parameters. Specifically we have shown that lipoic acid administered prior to induction of cirrhosis in the bile duct ligated rat can prevent the onset of the hyperdynamic circulation and improve renal function and that this corresponds to decreased nitric oxide formation as assessed by plasma nitrite+nitrate levels. In these experiments, lipoic acid is administered to cirrhotic rats *after* the induction of cirrhosis (7 days treatment) to assess whether lipoic acid can be used as a treatment for the hyperdynamic circulation.

Chapter 2: Effect of cirrhosis and/or endotoxaemia on platelet aggregation in rats: A role for S-nitrosothiols?

2.1. Introduction

The risk of variceal bleeding in cirrhotic patients is markedly increased following bacterial infection, but the mechanism remains unknown. It is well known that platelet aggregation is impaired in cirrhosis[159], but the effect of bacterial infection and endotoxaemia on platelet aggregation in cirrhosis has not previously been investigated. Since others have shown that S-nitrosothiols (RSNOs) inhibit platelet aggregation, and we have shown that injection of endotoxin leads to a marked increase in plasma RSNOs[217] in rats, I developed the hypothesis that endotoxaemia in cirrhosis during infection may lead to inhibition of platelet aggregation through upregulation of S-nitrosothiol synthesis. Furthermore, we and others have shown that low molecular weight thiols increase the rate of S-nitrosothiol decomposition *in vitro*[33] and lower plasma S-nitrosothiol concentration in rats following intraperitoneal injection in the form of the thiol containing antioxidant α-lipoic acid (unpublished data). It is therefore speculated that thiol antioxidants may improve dysfunctional platelet aggregation in liver disease, especially during infection.

The increased concentration of S-nitrosothiols previously reported in endotoxaemic cirrhotic rats by our group did not differentiate between S-nitrosothiol concentration and the mercury stable (non-S-nitrosothiol) plasma NO-carrying fraction[217]. Given that recent

reports have demonstrated that a large fraction of what was previously considered to be S-nitrosothiol species in physiological plasma samples are in fact non-mercury labile[133] it is important to re-evaluate the plasma S-nitrosothiol concentration in endotoxaemia and cirrhosis.

It is also known that platelets contain endothelial NOS (eNOS/NOSIII) and/or inducible NOS (iNOS/NOSII)[101,102]. Although platelets are enucleated, it is possible that NOS protein synthesis is elevated in the platelet progenitor cells, megakaryoctes, prior to platelet maturation in cirrhosis, resulting in increased platelet NO production in cirrhosis and lower platelet aggregation. It is also known that the NOS cofactor tetrahydrobiopterin (BH_4) is elevated in both cirrhosis and sepsis and can decrease platelet aggregation in platelets following pharmacological administration *in vivo*[104]. Incubation of isolated platelets with the general NOS enzyme inhibitor L-NAME is expected to attenuate any difference in platelet aggregation if platelet NOS is important in the mechanism of platelet dysfunction observed in cirrhosis and/or endotoxaemia.

The classical mechanism of NO and S-nitrosothiol mediated inhibition of platelet aggregation involves NO mediated stimulation of soluble guanylate cyclase and elevation of intraplatelet cGMP concentration[79,87]. However, it has also been shown recently that there is a cGMP independent mechanism of NO and S-nitrosothiol induced inhibition of platelet aggregation[88]. An investigation was therefore carried out in which isolated platelets were incubated with ODQ, a guanylate cyclase inhibitor, to assess whether the

dysfunction of platelet aggregation in cirrhosis and/or endotoxaemia is cGMP dependant or independent.

2.2. Materials and methods

All materials were sourced from Sigma, Poole UK unless otherwise stated.

2.2.1. Animals

All animal experiments were conducted according to Home Office guidelines under the Animals in Scientific Procedures Act 1986. Both Sprague Dawley and analbuminaemic rats were obtained from the Comparative Biology Unit at the Royal Free Hospital. Animals were used between 280-330g in weight and given free access to RM1 rat chow and water with a light cycle of 12 hours on and 12 hours off, at a temperature of 19 to 23°C, and a humidity of 50%.

2.2.1.1. Induction of biliary cirrhosis - Bile Duct Ligation (BDL)

Animals were anaesthetised under I.P. injection of diazepam (Durmex Ltd, Tring, UK) and I.M. injection of Hypnorm (Janssen Pharmaceuticals, Oxford, UK). The hair was shaved off in a $2cm^2$ area under the diaphragm and the exposed area swabbed with iodide. A 1.5cm midline incision was made just below the sternum and the duodenum was exposed using a blunt hook. The bile duct was located, cleaned and separated from the mesentery with blunt forceps. Three lengths of silk were then passed under the bile duct and each tied off with a quadruple knot. The bile duct was then cut between the two most cordal sutures. The incision was closed in two layers (parietal peritoneum and muscle layer, then skin) using 3-0 Vicryl sutures and the animal was allowed to recover

with free access to food and water. 2 hours post-operation each animal was given a subcutaneous injection of Temgesic (Schering-Plough, NJ, USA) for analgesia. Experiments were performed on days 24-26 post operation. Sham operations were carried out by the same method without tying off the bile duct.

2.2.1.2. Induction of endotoxaemia

The effects of endotoxaemia were investigated in normal and cirrhotic rats using a previously established model[215]. 0.5mg/Kg lipopolysaccharide (LPS) (Salmonella Typhimurium, Sigma) was injected to rats by intraperitoneal (I.P.) injection 2 hours prior to sacrifice. LPS was made up in sterile 0.9% saline and stored at 4°C.

2.2.1.3. Administration of low molecular weight thiol containing antioxidants

2.2.1.3.1. Lipoic acid (LA)

Lipoic acid was administered via intraperitoneal (I.P.) injection. Lipoic acid was administered at 100mg/Kg/day for 7 days (days 18-24 post-bile duct ligation) and was prepared by dissolving in a minimum volume of 1M NaOH, made up to volume in distilled water, and titrated back to pH 7.0 with 1M HCl. Before injection, the resulting preparation was filtered through a 0.45um filter to remove particulate matter and micro-

organisms. Generally the approximate [NaOH] was 1mM which, given a typical injection volume of 1mL/day, which equates to 23μg sodium/day.

2.2.1.3.2. N-acetylcysteine (NAC)

N-Acetylcysteine (NAC) was supplied ready for injection (Parvolex, Evans Medical Ltd., Leatherhead, UK). NAC was administered by subcutaneous (s.c.) injection twice daily at a dose of 100mg/Kg/day for 7 days (days 18-24 post-bile duct ligation). The final injection was made on the day of the final experiment, at least 2 hours before final experiment.

2.2.2. Histology

Liver samples were fixed in formalin and embedded in paraffin blocks. Sections 3μm thick were cut and stained with haematoxylin and eosin. Slides were examined by a qualified histopathologist and scored for architectural changes, fibrosis and cirrhosis (Ishak scoring).

2.2.3. Liver function tests

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were used to assess hepatocyte injury. As a result of hepatocyte injury in liver disease, the synthesis of albumin is impaired and bilirubin accumulates in the circulation. The concentration of bilirubin also increases with biliary obstruction, as in our model of bile duct ligation. Plasma ALT, AST, albumin and bilirubin concentration were determined by auto-analyser (Hitachi, UK)

2.2.4. Plasma thiol concentration

Plasma sulphydryl concentration was analysed using a newly validated adaptation of the classic Ellman's assay[243] which controlled for absorbance of plasma constituents, notably bilirubin, at 412nm (see below).

2.2.4.1. Reagents

Plasma samples were thawed on ice and were stored on ice until assay. 20mM Dithionitrobenzene (DTNB) was dissolved in methanol and used within 3 months upon storage at 4°C. 0.1M potassium phosphate buffer, pH 8.0 was made up on the day of use. GSH standards were made up on the day of analysis in 0.1M potassium phosphate, pH 8.0 buffer. Standards were prepared from 100μM to 1μM along with a buffer blank.

2.2.4.2. Reaction

Two cuvettes were prepared for each sample as below.

Cuvette 1 - sample cuvette:

825μL 0.1M potassium phosphate buffer, pH 8.0

125μL plasma

50μL 20mM DTNB.

Cuvette 2 - blank cuvette:

875μL 0.1M potassium phosphate buffer, pH 8.0

125μL plasma

NB. The absorbance of buffer and methanol at 412nm is not significantly different

Standards were prepared as below and the absorbance of the buffer was also determined.

Standards:

825μL 0.1M potassium phosphate, pH 8.0

125μL GSH standard (0μM {phosphate buffer} 15, 31, 62.5, 125, 250, 500 and 1000μM)

50μL DTNB

Buffer:

1000μL 0.1M potassium phosphate, pH 8.0

The absorbance in each tube can be therefore considered thus:

Cuvette 1: $Absorbance_{412} = Plasma_{412} + Buffer_{412} + Unreacted\ DTNB_{412} + TNB_{412}$

Cuvette 2: $Absorbance_{412} = Plasma_{412} + Buffer_{412}$

Standards: $Absorbance_{412} = Buffer_{412} + Unreacted\ DTNB_{412} + TNB_{412}$

Buffer: $Absorbance_{412} = Buffer_{412}$

Therefore, in order to compare the absorbance of TNB produced from reaction with thiol in plasma against standards, the absorbance from plasma must be subtracted.

1. Absorbance of Cuvette 1 – Absorbance of Cuvette 2:

$$Absorbance_{412} = Unreacted\ DTNB_{412} + TNB_{412}$$

Therefore, by subtracting the absorbance of cuvette 2 from that of cuvette 1 and then adding the absorbance of buffer the absorbance value obtained can be compared to standards:

2. Absorbance$_{412}$ of Cuvette 1 – Absorbance$_{412}$ Cuvette 2 + Absorbance$_{412}$ Buffer:

$$\text{Absorbance}_{412} = \text{Buffer}_{412} + \text{Unreacted DTNB}_{412} + \text{TNB}_{412}$$

2.2.4.3. Detection

The absorbance at 412nm was recorded on a Kontron Instruments spectrophotometer (Kontron Instruments, Bletchley UK).

2.2.4.4. Validation of assay

Due to the possibility of interference from plasma proteins, notably bilirubin in cirrhotic samples, the sensitivity and specificity of the assay was determined in both control and cirrhotic plasma by adding known amounts of N-acetylcysteine (NAC). The slope of the standard curve obtained after addition of known amounts of NAC in plasma (0, 235 and 462μM) was compared to that of GSH standards in buffer. The linearity of the relationship between spike dose and increase in absorbance was also compared by measurement of the square of the Pearson product moment correlation coefficient (R^2).

The slope of the GSH standards was $1.8\pm0.03\times10^{-3}$ compared to $1.7\pm0.1\times10^{-3}$ for the spiked plasma (Figure 11). The difference between these was not statistically significant ($p=0.086$, as analysed by parametric students t-test) but the mean slope for the NAC spiked plasma standards was only 5% lower than that for the GSH standards (Figure 11). Also the linearity of the standard curve produced in all experiments (with or without plasma) was excellent: $R^2>0.99$ in all experiments (regression analysis by Pearson regression analysis). It is therefore concluded that it is possible to use this assay to determine plasma thiol concentration at least up to 462μM if the interference from the plasma itself is removed (see above).

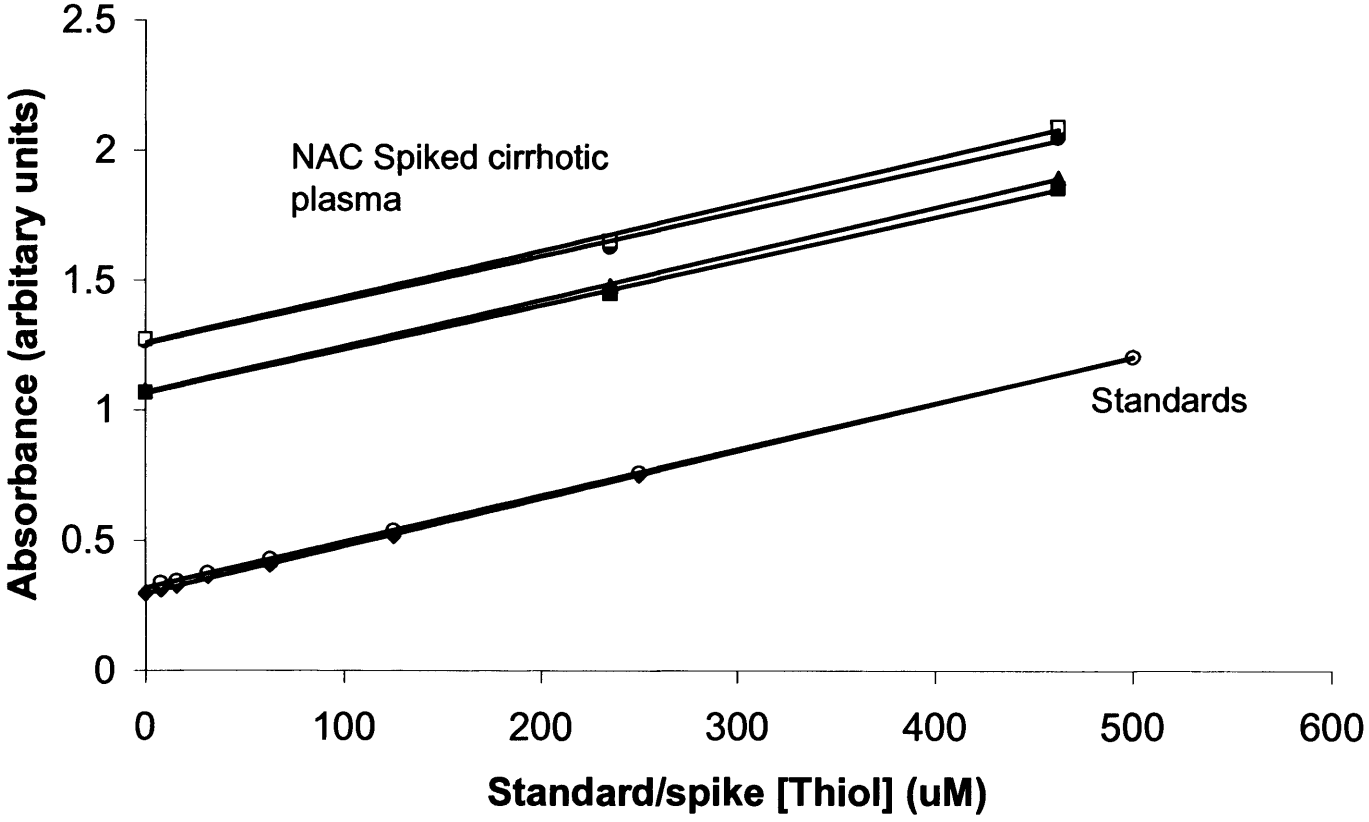


Figure 11. Absorbance recorded following addition of known quantities of GSH to buffer (standards) and known quantities of NAC to cirrhotic plasma. Similar data was demonstrated in normal plasma (not shown). The slope of the NAC spiked plasma was the same as GSH standards indicating that interference from plasma proteins, notably bilirubin, in plasma samples can be controlled for.

2.2.5. Nitrite+Nitrate

Reagents: Aliquots of plasma were removed from the -80°C freezer and thawed on ice. Tris/HCl (20mM), pH 7.6 buffer was made up freshly on the day of use. Sodium nitrate standards were made up freshly on the day of use. Frozen aliquots of 100mM sodium nitrite were defrosted and serially diluted with MilliQ water to give concentrations of 100μM, 50μM, 25μM, 10μM, 5μM, 2.5μM, 1μM and 0μM standards.

Reduction of nitrate: Nitrate was reduced to nitrite for analysis by the incubation of sample/standard with 80μM NADPH, 8μM FAD and 0.04 U/mL nitrate reductase in 20mM Tris/HCl, pH 7.6 buffer. The assay was carried out in Eppendorf tubes. Nitrate reductase was added as the final reagent and samples and standards were left at room temperature for one hour to react.

Detection of nitrite: The concentration of nitrite was determined by chemiluminescence using a Sievers 280 NOA (nitric oxide analyser, Sievers Research Inc, Boulder CO) as per previous studies[215,217]. After incubation, the samples and standards were diluted 1:10 with MQ water directly before injection into a purge vessel containing 8mL glacial acetic acid and 2mL KI. The purge vessel was connected in line to the NOA and was constantly purged with nitrogen. The acid/iodide mixture reduces the nitrite to NO which is detected from the chemiluminescent reaction of NO with ozone. Data was recorded using Sievers

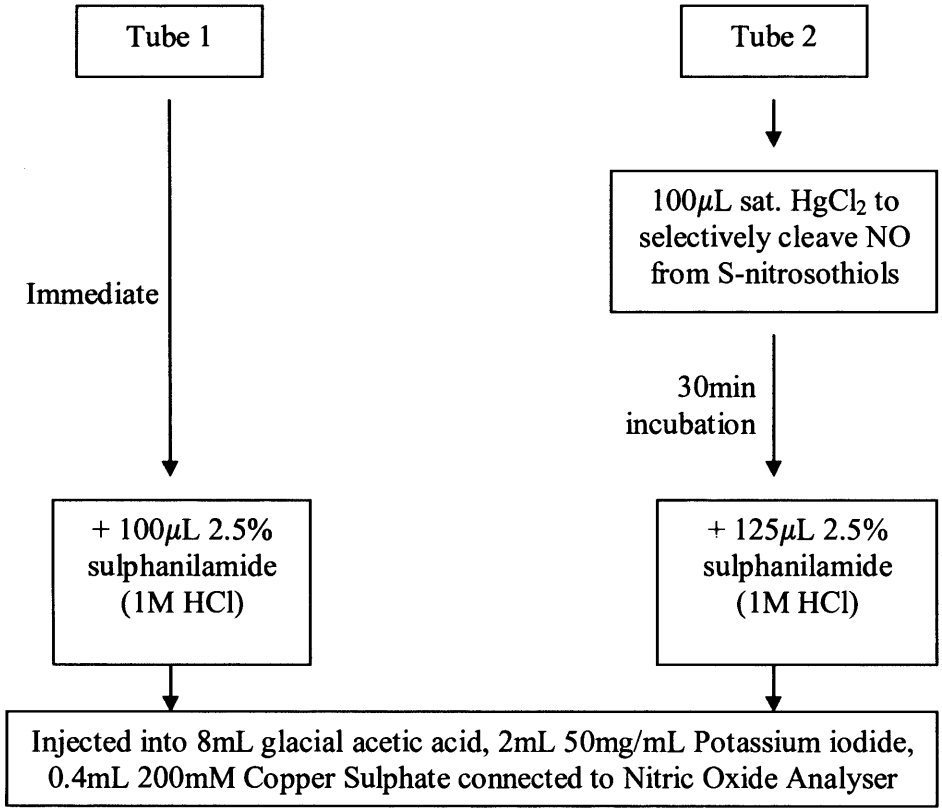
"liquid" software and analysed with Origin 6.1 data analysis software (Origin Corporation, Northampton, MA)

2.2.6. Measurement of S-nitrosothiol/mercury-stable NO-carrying species concentration

Measurement of plasma S-nitrosothiol concentration was carried out by chemiluminescent detection of NO liberated from S-nitrosothiols and mercury-stable NO-carrying species (HgSNOCS) as described by Yang *et.al.*[132]

Preparation of plasma: Animals were anaesthetised with pentobarbital and exsanguinated through the abdominal aorta using a 23G butterfly needle. Blood was collected into a 5mL syringe containing 20U heparin and two 900μL aliquots added to two eppendorf tubes containing 50μL 200mM N-ethyl-maleimide (0.9% NaCl) and 50μL 2mM DTPA (0.9% NaCl) and inverted to mix. The tubes were then spun at 1200*g* directly for 1min and the plasma removed for immediate analysis.

Two 400μL aliquots of NEM/DTPA plasma were placed in separate tubes:



Analysis: The samples from tube 1 and tube 2 were injected into a purge vessel (purged with a steady flow of nitrogen) containing 8mL glacial acetic acid, 2mL 50mg/mL KI and 0.4mL 200mM $CuSO_4$ which was connected in line to a Sievers 280 NOA (Nitric Oxide Analyser, Sievers Research Inc, Boulder CO). The signals recorded were analysed against nitrite standards from 2.5μM to 3.125nM. The signal was recorded using Sievers "liquid" software and analysed using Origin 6.1 software (Origin software was used rather than Sievers "liquid" software as Origin allows peak smoothing and multi-point adjustment of the baseline). This facilitates interpretation of lower peak heights and

therefore higher assay sensitivity. The signal from tube 1 was the signal from the S-nitrosothiol fraction of NO carrying species plus the signal from the mercury non-decomposable fraction. The signal from tube 2 was the signal from the mercury non-decomposable fraction of plasma NO carrying species.

2.2.7. Measurement of platelet aggregation

Preparation of platelet rich plasma: Animals were anaesthetised with pentobarbital and exsanguinated through the abdominal aorta using a 23G butterfly needle. Blood was collected into a 5mL syringe and 4.5mL added to 0.5mL 3.8% Tri sodium citrate. The blood was then spun at 820rpm at room temperature (RT) for 10 minutes to obtain PRP. 1mL of platelet rich plasma (PRP) was removed and the remaining blood spun at 3000rpm for 10mins, RT to obtain platelet poor plasma (PPP). PRP was analysed using an ADVIA 120 electronic particle analyser (Bayer Diagnostics, Newbury UK) to determine platelet count. PRP was diluted to 200 000 platelets/μL with PPP for analysis of aggregation. PRP and PPP were then left at room temperature for 10mins before analysis.

Analysis of platelet aggregation: Aggregation was followed by measurement of light transmission using a Chronolog platelet aggregometer (Chronolog Instruments, Havertown, PA). 240μL aliquots of PRP and 250μL aliquots of PPP were placed in 1mL cuvettes in the 37°C heating blocks of the aggregometer for 10mins. Disposable magnetic stir bars were then added to the PRP and the output from the aggregometer was recorded

using bundled Chronolog software. PRP was zeroed with respect to PPP and 10μL of 2mM, 200μM, 50μM or 20μM ADP was added to initiate platelet aggregation (80, 8, 2 and 0.8μM ADP final concentration respectively). The raw data from the bundled Chronolog software was exported to Graphpad Prism (San Diego, USA) and this software was used to analyse the area under the curve for the profile of platelet aggregation. The area under the curve was then plotted against [ADP] to get an ADP conencentration response curve.

2.2.7.1. Incubation of platelets with L-NAME

L-NAME was dissolved in PBS on the day of use and used within 2 hours of dissolution. L-NAME was stored at 4°C until use. To 240μL aliquots of PRP in cuvettes was added 10μL of either 750μM or 2.5mM L-NAME to give a final concentration of 30μM or 100μM L-NAME respectively. To another 240μL aliquot of PRP was added 10μL PBS. These aliquots of PRP were incubated at room temperature for 20 minutes and then transferred to the 37°C incubation chambers of the platelet aggregometer for 10 minutes. Magnetic stir bars were added for the final 3 minutes. After the 10 minute 37°C incubation period platelet aggregation was initiated by addition of 10μL 200μM ADP (8μM final concentration)

2.2.7.2. Incubation of platelets with ODQ

ODQ was dissolved in dimethysulphoxide (DMSO) to give a 100mM solution. The 100mM ODQ was then serially diluted in PBS to produce 750μM and 250μM ODQ solutions. Aliquots were frozen and stored at -80°C and used within 1 month.

Platelet rich plasma (PRP) was isolated from endotoxaemic cirrhotic rats as described above. 10μL of either 750μM or 250μM ODQ were added to 240μL of PRP in a cuvette. A control experiment was set up in which an equal 10μL volume of DMSO/PBS vehicle was added to a further 240μL aliquot of PRP. An experiment in which 10μL PBS was added to a 240μL aliquot of PRP was also set up. These aliquots of PRP were then left to incubate at room temperature for 20 minutes. After 20 minutes incubation at room temperature the cuvettes were transferred the 37°C incubation chambers of the platelet aggregometer for 10 minutes. Magnetic stir bars were added for the final 3 minutes. After the 10 minute 37°C incubation period platelet aggregation was initiated by addition of 10μL 200μM ADP (8μM final concentration)

2.3. Results

2.3.1. Establishment of biliary cirrhosis in the rat

Histological assessment and biochemical liver function tests were used to determine the degree of liver dysfunction in the bile duct ligated rat as per previous studies[229]

2.3.1.1. Histology

No liver samples from sham operated rats showed fibrosis but liver samples from BDL cirrhotic rats had an Ishak score of 5 (n=3 for all groups). This signifies "Marked bridging (portal to portal and or portal-central) with occasional nodules (incomplete cirrhosis)"[244]. This confirms previous data on the BDL model of cirrhosis over 24 days. Injection of LPS has no effect on fibrosis in either sham operated or BDL cirrhotic rats.

2.3.1.2. Liver function tests

The plasma concentration of AST and ALT both rose significantly after induction of cirrhosis as expected (Fig.12, $p<0.001$ and $p<0.05$ respectively). 2Hr. injection of LPS to normal or cirrhotic rats had no statistically significant effect on plasma [ALT]. However, the increase in plasma [AST] in normal and cirrhotic rats following injection of LPS was significant ($p<0.05$ in both groups, Fig.12). Therefore, LPS also releases

aminotransferase enzyme into the circulation as well as induction of cirrhosis, probably as a result of inflammation and necrotic cell death.

Plasma albumin levels fell in cirrhosis and plasma bilirubin also increased sharply, indicative of hepatocellular damage (see Fig.13). Injection of LPS had no effect on these parameters in either normal or cirrhotic rats as expected.

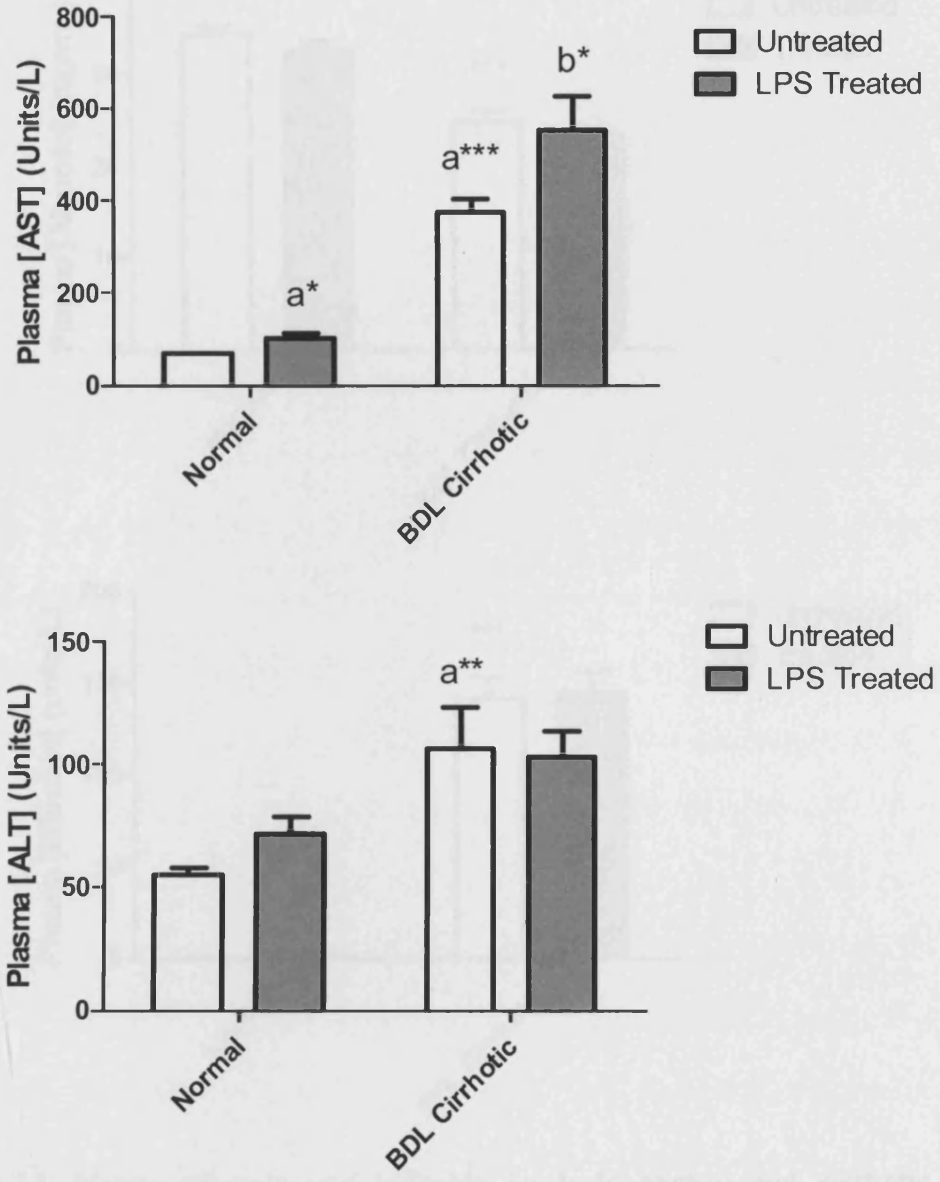


Figure 12. Assessment of liver function by analysis of plasma transaminase enzyme activity (ALT and AST) in Sprague Dawley rats±cirrhosis±LPS induced endotoxaemia. There was a significant rise in both plasma AST and ALT concentrations following induction of cirrhosis by bile duct ligation over controls as assessed by two-way ANOVA ($P<0.0001$ for both AST and ALT), indicating the release of these enzymes from the liver through hepatocellular necrosis. The rise in [AST] induced by injection of LPS to sham operated rats was significant for AST ($P=0.02$)). Post-hoc test comparisons carried out by Newman-Keuls method: *$p<0.05$, **$p<0.01$ vs. control ($n \geq 5$ for each group). Parametric statistical analysis was carried out after logarithmic transformation of data.

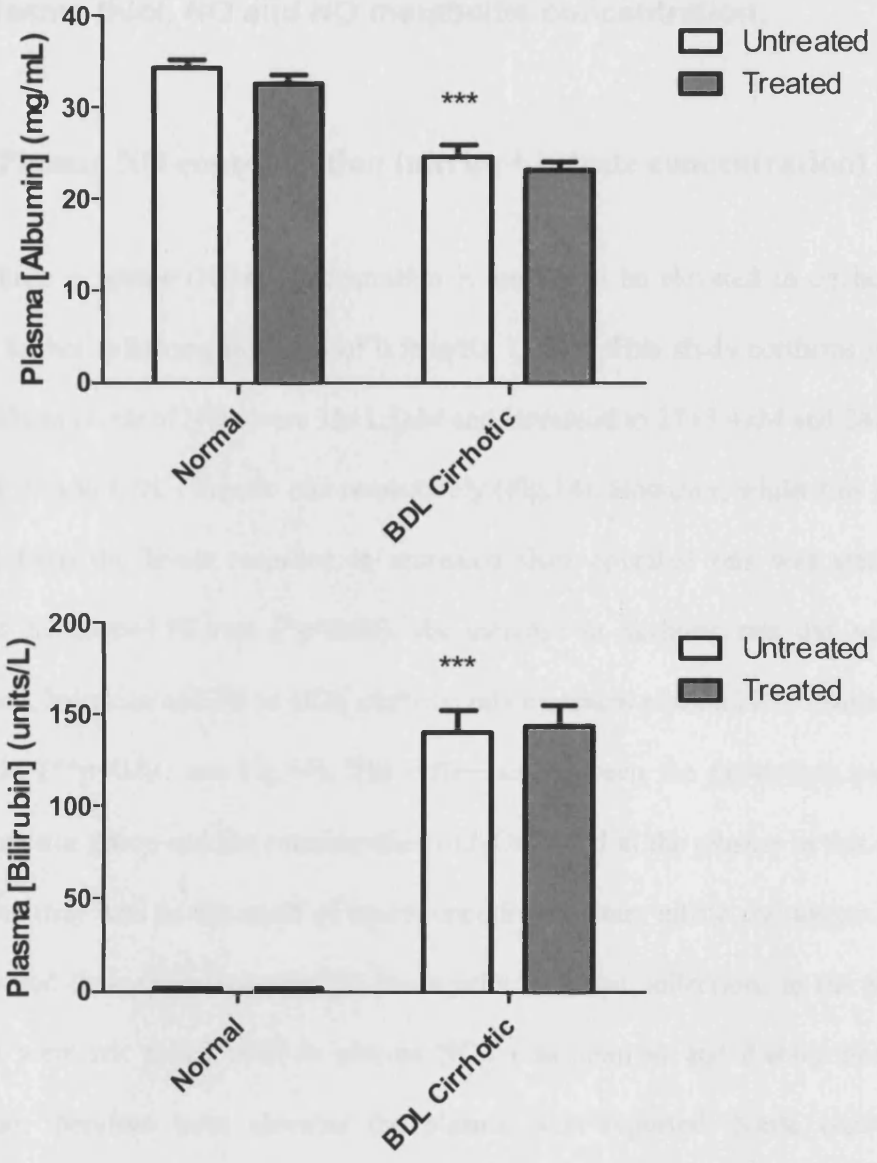


Figure 13. Plasma albumin and bilirubin levels in normal and cirrhotic rats. Albumin concentration is lower in cirrhotic rats than in normal rats ($P<0.0001$) whilst plasma bilirubin concentration increased following induction of cirrhosis ($P<0.0001$), consistent with impaired liver function (statistical analysis by two-way ANOVA). 2Hr. injection of LPS had no effect on either plasma albumin or bilirubin concentration ($P=0.132$ and $P=0.818$ respectively). ***$P<0.0001$ vs. untreated normal controls. Post-hoc test comparisons carried out by Newman-Keuls method: ***$p<0.001$ ($n \geq 5$ for each group).

2.3.2. Plasma thiol, NO and NO metabolite concentration.

2.3.2.1. Plasma NO concentration (nitrite + nitrate concentration)

Plasma nitrite + nitrate (NOx) concentration is known to be elevated in cirrhosis and increases further following injection of 0.5mg/Kg LPS[215]. This study confirms previous findings. Sham levels of NOx were 15±1.3μM and increased to 27±3.4μM and 24±3.4μM in sham+LPS and BDL cirrhotic rats respectively (Fig.14). However, whilst this increase in [NOx] from the levels recorded in untreated sham-operated rats was statistically significant in sham+LPS rats (*$p<0.05$), the increase in cirrhotic rats did not reach significance. Injection of LPS to BDL cirrhotic rats increases plasma NOx concentration to 45±7μM (**$p<0.01$, see Fig.14). The difference between the previously published results from our group and the concentration of NOx found in the plasma in this study is striking and may well be the result of interference from dietary nitrite and nitrate. Rats in this study had their chow removed 24 hours prior to blood collection. In the previous study rats were not fasted prior to plasma NOx measurement and dietary nitrite and nitrate may therefore have elevated the plasma NOx reported. Nitric oxide does, however, appear to be elevated in cirrhosis and further elevated following LPS injection to cirrhotic rats. Unfortunately, there was insufficient plasma remaining from NAC treated BDL+LPS rats to measure NOx concentration However, previous studies from our lab have demonstrated a fall in NOx concentration in BDL rats following lipoic acid administration[229].

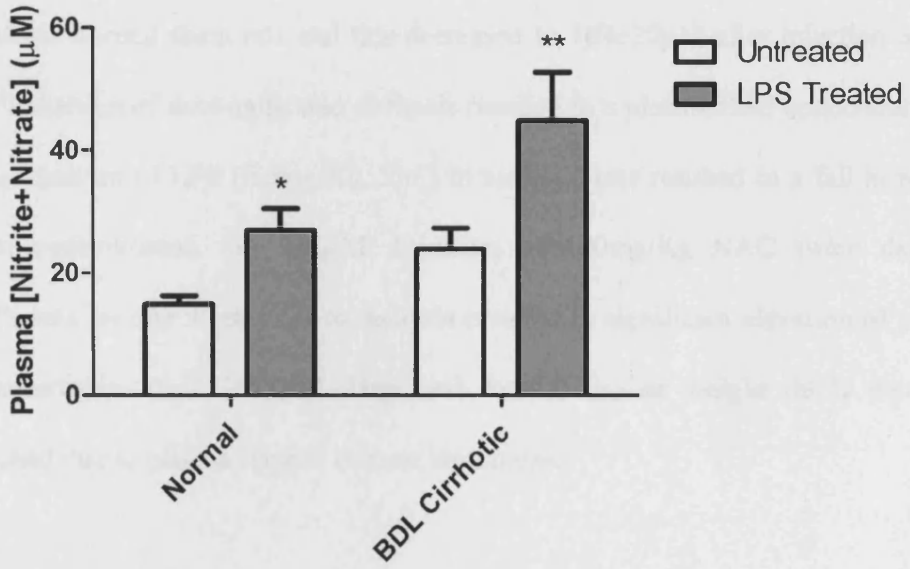


Figure 14. Plasma nitrite+nitrate (NOx) concentration in the plasma of Sprague Dawley rats±cirrhosis±LPS. The increase in NOx concentration is indicative of increased NO production. NO production in normal rats is increased above basal levels to a roughly equal degree by injection of 0.5mg/Kg LPS or by induction of cirrhosis. However, whilst this increase was significanct in LPS treated rats ($p<0.05$), the increase in [NOx] following induction of cirrhosis did not reach significance. Injection of LPS to BDL cirrhotic rats significantly increased NO production above basal level in BDL rats ($p<0.01$). *$p<0.05$ vs. untreated normal rats, **$p<0.01$ vs. untreated BDL cirrhotic rats. $n \geq 8$ in all groups. Statistical analysis was carried out after logarithmic transformation of data. Two-way parametric ANOVA was applied with Newman-Keuls post-hoc paired comparisons (***$p=0.0003$).

2.3.2.2. Plasma thiol concentration

Total plasma free thiol (reduced sulphydryl) concentration was evaluated using an adaptation of the Ellman reaction. Total plasma thiol concentration was found to be 238±14μM in normal sham rats and this decreased to 169±20μM after injection of LPS (Fig.15). Induction of uncomplicated cirrhosis resulted in a plasma thiol concentration of 60±9μM. Injection of LPS (0.5mg/Kg, 2hr.) to cirrhotic rats resulted in a fall in plasma free thiol concentration to 19±2μM. Injection of 100mg/Kg NAC twice daily to BDL+LPS rats for one week prior to analysis resulted in significant elevation of plasma thiol concentration to 151±58μM. High and low molecular weight thiols were not distinguished due to plasma sample volume limitations.

The major circulating plasma thiol is albumin which carries one vicinal thiol per molecule and is present in the circulation at ~35g/L in healthy humans and rats. This presents a theoretical thiol concentration of ~500μM assuming a molecular weight of 68KDa and complete reduction of all vicinal thiol groups. Thus, less than 50% of plasma thiols are present in their reduced form in normal rats, which is expected due to the prevalence of mixed disulphides in the plasma and the role of the vicinal thiol of albumin in attachment of transported molecules. The 29% fall in plasma free thiol concentration following injection of LPS is probably the result of oxidation of thiol groups of albumin together with plasma dilution. However, the dramatic 75% decrease in plasma free thiol concentration in BDL cirrhotic rats was unexpected. The reasons for the low concentration of free thiol detected in the plasma of cirrhotic rats could be the result of an

oxidation of the circulating thiols in cirrhotic rats. This is supported by the observation that whilst cirrhotic rats injected with LPS have even lower plasma concentration of free thiol than cirrhotic rats (18.7±2.0μM vs. 60±9 μM), twice daily injection of NAC for one week prior to analysis restores plasma free thiol concentration to 151±58μM: 63% of normal sham operated levels. Plasma protein thiol redox status could therefore be sacrificed in conditions of chronic oxidative stress as thiol antioxidants are consumed maintaining critical intracellular redox status and plasma low molecular weight antioxidants.

The effect of oxidation of plasma protein thiols in cirrhosis is potentially profound. Many plasma proteins are sensitive to thiol redox status including those involved in the haemostatic system. A recent study by Bayele et.al. has demonstrated that alteration of thiol redox status can affect prothrombin time (PT) as well as activated partial thromboplastin time (APTT) in human platelets[245]. The authors note that disulphide interchange is necessary for the function of fibrinogen[246] and thrombospondin[247]. They also note that the protein disulphide isomerase (PDI, a dithiol containing protein involved in numerous thiol redox reactions) expression and redox status on the platelet surface is necessary for platelet aggregation[46].

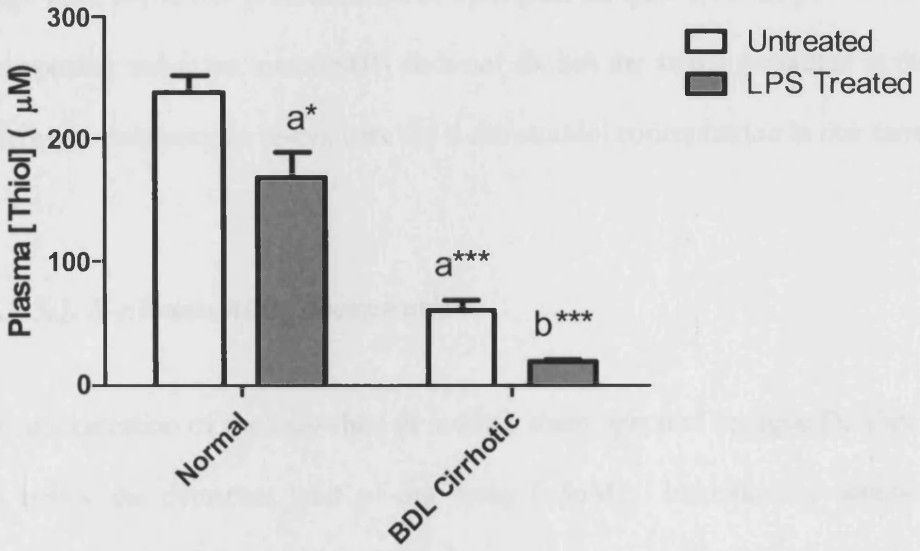


Figure 15. Plasma reduced thiol concentration in normal and cirrhotic rats. Reduced thiol concentration is decreased by 29% in normal rats when injected with LPS compared to a decrease of 75% when cirrhosis is induced by bile duct ligation. Injection of LPS to cirrhotic rats resulted in a dramatic reduction in reduced plasma thiol concentration to 18.7μM, 7% of the basal level found in normal rats. Statistical analysis was carried out after logarithmic transformation of data. Two-way parametric ANOVA was applied with Newman-Keuls post-hoc paired comparisons. a = vs. untreated normal, b = vs. untreated BDL cirrhotic, *$p<0.05$, ***$p<0.001$. $n \geq 3$ in each group

2.3.2.3. Other NO metabolite concentrations

The concentration of S-nitrosothiols previously published by our group may have been an overestimate since we did not use Hg^{2+} decomposition to verify the structure. Other groups have found that preincubation of biological samples with the potent S-nitrosothiol decomposing metal ion mercury(II) does not abolish the signal generated in our assay. It is therefore necessary to re-evaluate the S-nitrosothiol concentration in our samples.

2.3.2.3.1. S-nitrosothiol concentration

The concentration of S-nitrosothiol in normal, sham operated Sprague Dawley rat plasma was below the detection limit of our assay (<5nM). Induction of endotoxaemia by injection of 0.5mg/Kg LPS, 2Hr. to sham operated rats or induction of cirrhosis by bile duct ligation did not elevate S-nitrosothiol concentration above the detection limit (Table 6). A nominal value of 8.3±8.3nM S-nitrosothiol concentration is reported for Sham + LPS rats as 33nM S-nitrosothiol was detected in one rat but no S-nitrosothiol was detected in any other individuals. Injection of 0.5mg/Kg LPS, 2Hr. to BDL cirrhotic rats resulted in the generation of 659±209nM S-nitrosothiols. However, injection of 100mg/Kg NAC to BDL+LPS rats for 1 week prior to analysis did not significantly lower the plasma S-nitrosothiol concentration as expected 493±196nM ($p=0.58$ vs. BDL+LPS untreated), see Fig.16.

The previous S-nitrosothiol concentrations in normal, cirrhotic and/or endotoxaemic rats reported by our group were much higher than those found in this study, see Table 6. However, this does not take into account the concentration of mercury-stable NO-carrying species (HgSNOCS) found in the present study which were not previously differentiated. However, other groups as well as ours (personal communication) have noted that plasma S-nitrosothiol levels recorded, particularly under physiological conditions, have decreased over time using this assay possibly through increased familiarity with the assay.

	Previous results	**This study**
Sham	51±6nM	0
Sham + LPS	206±59nM	8.3±8.3nM
BDL Cirrhotic	108±23nM	0
BDL Cirrhotic + LPS	1335±423nM	659±209

Table 6. Comparison of plasma S-nitrosothiol concentrations reported in previous and present study. In contrast to the previous study[217] significant concentrations of plasma S-nitrosothiols (RSNO) could not be consistently detected in the plasma of normal rats or rats with cirrhosis induced by bile duct ligation. 33nM S-nitrosothiol concentration was detected in the plasma of one normal rat injected with LPS but no significant S-nitrosothiol could be detected in any other individuals (n=4). However, injection of LPS to cirrhotic rats resulted in 659±209nM plasma S-nitrosothiol concentration. n ≥3 for all groups. n=7 for cirrhotic + LPS.

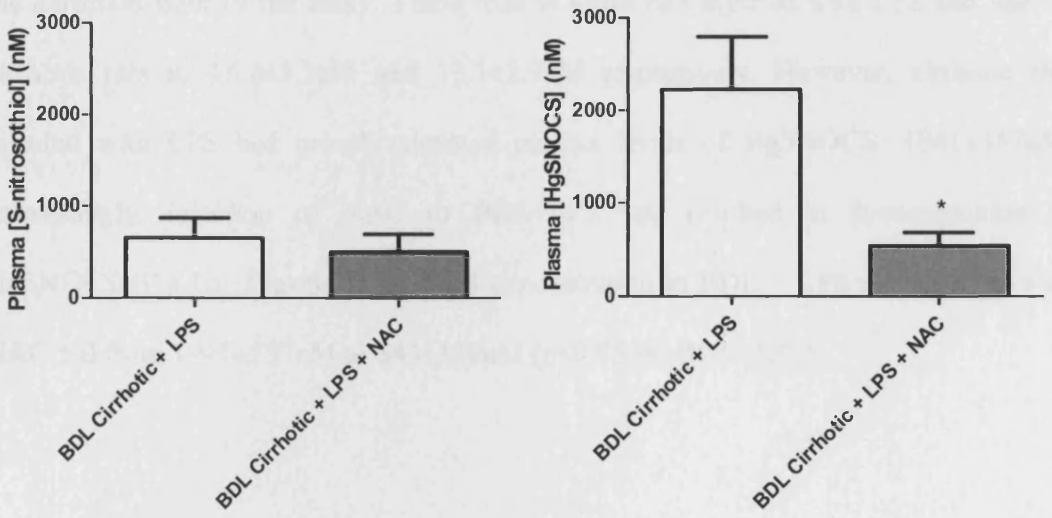


Figure 16. Effect of 100mg/Kg/day NAC injection on plasma S-nitrosothiol and HgSNOCS concentration in endotoxaemic BDL cirrhotic rats. Whilst plasma S-nitrosothiol concentration was not affected by injection of NAC, Plasma mercury-stable-NO-carrying species (HgSNOCS) concentration was significantly reduced. * $p<0.05$ n=7 BDL+LPS, n=6 BDL+LPS+NAC. Statistics carried out by non-parametric unpaired two-tailed Mann-Whitney t-test.

2.3.2.3.2. Mercury-stable NO-carrying species (HgSNOCS) concentration

Mercury-stable NO-carrying species (HgSNOCS) were found in greater concentrations than S-nitrosothiols under all conditions (Table 7). Sham levels were 4.7±2nM, around the detection limit of the assay. These rose in sham rats injected with LPS and also in cirrhotic rats to 16.2±9.3nM and 13.1±2.9nM respectively. However, cirrhotic rats injected with LPS had grossly elevated plasma levels of HgSNOCS: 1941±557nM. Interestingly, injection of NAC to BDL+LPS rats resulted in decomposition of HgSNOCS (Fig.16). Plasma HgSNOCS concentration in BDL + LPS rats injected with NAC fell from 1941±557nM to 543±130nM ($p<0.05$ vs. BDL+LPS).

Group	**S-nitrosothiol (Hg-labile fraction)**	**Hg-stable component**
Sham	trace	5±2nM
Sham + LPS	trace	16±9nM
Cirrhotic	0nM	13±3nM
Cirrhotic + LPS	660±210nM	1941±557nM**

Table 7. S-nitrosothiol and HgSNOCS concentration in the plasma of normal and cirrhotic Sprague Dawley rats with or without lipopolysaccharide (LPS) or N-acetylcysteine (NAC) injection. Low nanomolar concentrations of HgSNOCS were found in all groups, but were found at dramatically higher levels (1.9μM) in endotoxaemic cirrhotic rats. n≥3 for all groups. n=7 for cirrhotic + LPS. The concentration of HgSNOCS was markedly elevated in cirrhotic rats injected with LPS compared to untreated cirrhotic rats. Statistical analysis by two-way ANOVA ($P<0.0001$) with Newman-Keuls post-hoc paired comparison, **$p<0.01$.

2.3.3. Platelet aggregation – Concentration response to ADP induced aggregation

2.3.3.1. Sham operated rats:

The maximum platelet aggregation in sham operated rats increased upon stimulation with increasing concentrations of ADP to a maximum aggregation observed at 8μM, see fig.17. Stimulation of platelets with 80μM ADP did not result in any greater maximum stimulation of platelet aggregation.

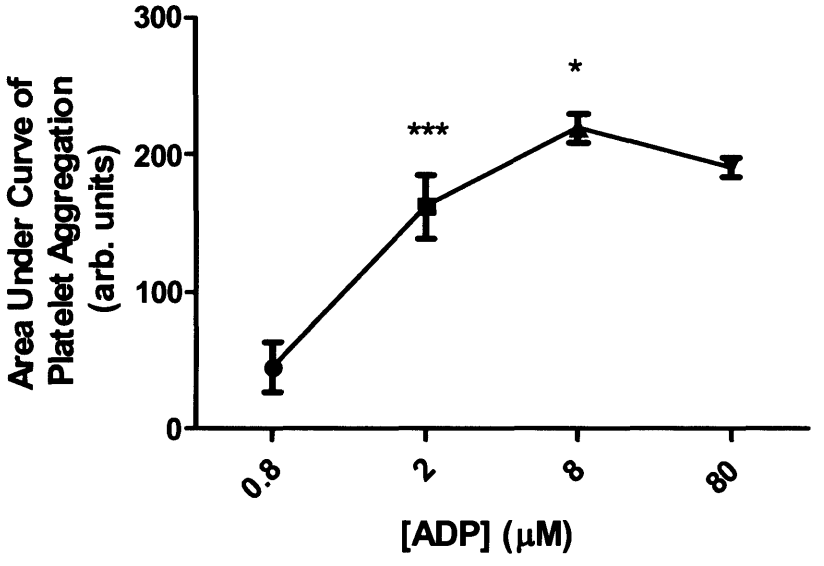


Figure 17. Dose response curve for platelet aggregation in sham operated rats stimulated with increasing concentrations of ADP. Platelet aggregation increased in the order 0.8< 2 < 8μM. However, 80μM ADP induced slightly less platelet aggregation than 8μM ADP, possibly due to a desensitisation mechanism at this concentration. n=6 for all ADP concentrations. Statistical analysis by parametric one-way ANOVA (P<0.0001) with Newman-Keuls post-hoc paired comparison. ***p<0.001 vs. 0.8μM, *p<0.05 vs. 2μM.

2.3.3.2. Cirrhotic rats and endotoxaemic rats

Either induction of cirrhosis by bile duct ligation in normal Sprague Dalwey rats, or induction of endotoxaemia by injection of 0.5mg/Kg LPS to normal Sprague Dawley rats resulted in an almost identical drop in platelet function when aggregation was stimulated with 0.8-8μM ADP, see Fig.18. The dysfunction of platelet aggregation was statistically significant at 2μM ADP concentrations in both groups ($p<0.001$ BDL cirrhotic and $p<0.01$ Normal+LPS) and in LPS treated rats at 8μM concentration ($p<0.01$). However, 80μM ADP stimulated PRP from sham, sham+LPS and cirrhotic rats resulted in the same platelet aggregation indicating that sufficient stimulation could overcome the dysfunction in aggregation in either cirrhotic or endotoxaemic rats.

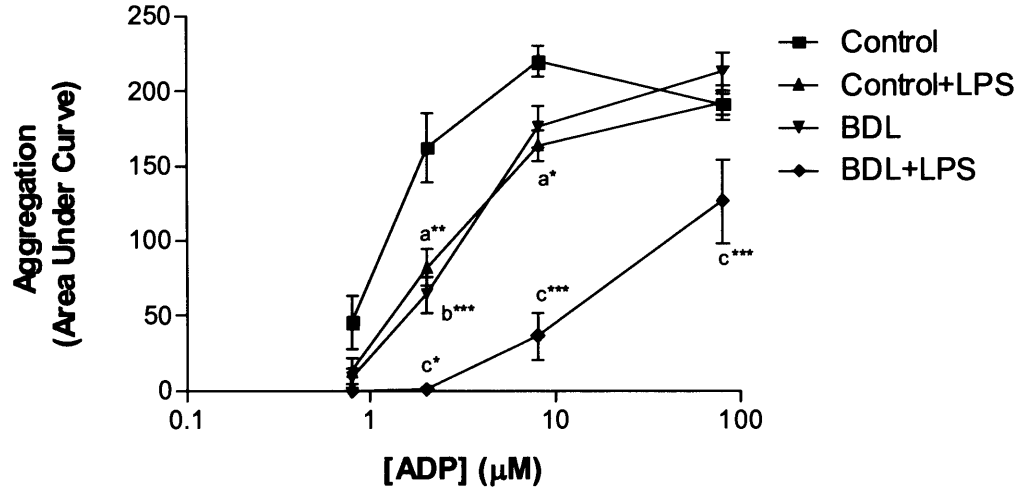


Figure 18. Platelet aggregation in normal and cirrhotic rats ± endotoxaemia. Platelet aggregation increased as the stimulating concentration of ADP increased in all groups ($P<0.0001$). Platelet aggregation was impaired in all groups compared to sham controls at 2μM ADP concentrations and in LPS treated and BDL+LPS treated animals at 8μM concentration. At 80μM ADP concentration, the impairment of platelet aggregation in Sham+LPS and BDL cirrhotic rats observed at lower concentration was overcome, but was still observed in BDL cirrhotic + LPS rats. Overall, platelet aggregation in BDL+LPS rats was drastically impaired at all concentrations. Statistics performed using two-way ANOVA with Neman-Keuls post-hoc comparison. a=Control+LPS compared to sham, b=BDL cirrhotic compared to sham, c=BDL cirrhotic + LPS compared to BDL cirrhotic. *=$p<0.05$, **=$p<0.01$, ***$p<0.001$. n=6 for Sham and Sham+LPS, n=7 for BDL and n=5 for BDL+LPS

2.3.3.3. Endotoxaemic cirrhotic rats

It was hypothesised that endotoxaemia in cirrhosis may lead to platelet dysfunction and that this may explain the increased risk of increased variceal bleeding in cirrhotic patients with infections. The induction of endotoxaemia in cirrhotic rats by injection LPS caused a large decrease in platelet aggregation, see fig.18. This was significant at all concentrations of ADP used, except 0.8μM, at which concentration no significant aggregation was observed. At 8 and 80μM ADP stimulation concentrations the induction of endotoxaemia in cirrhotic rats caused a dysfunction of platelet aggregation, greater than the sum of the constituent dysfunction caused by cirrhosis and endotoxaemia separately, see fig.18.

The observation that 80μM ADP stimulation of both BDL cirrhotic and sham+LPS PRP resulted in restoration of aggregation to sham levels shows that with sufficient stimulation full aggregation can be restored. This suggests that the dysfunction in platelet aggregation observed in BDL cirrhotic and sham+LPS rats may be predominantly a problem in the signal transduction cascade to initiate granular release (secondary aggregation). This contrasts to the observaion that aggregation in 80μM ADP stimulated platelets from BDL+LPS rats only partially improves platelet aggregation with significant inhibition still being observed. The fact that endotoxaemia and cirrhosis effects platelet aggregation more than the sum of endotoxaemia or cirrhosis alone is may be explained by these conditions affecting platelet activation and primary aggregation rather than just initiation of secondary aggregation.

2.3.4. Effect of low molecular weight antioxidant administration on platelet aggregation in endotoxaemic cirrhotic rats

Either lipoic acid (LA, 100mg/Kg/day) or NAC (100mg/Kg/day) were administered I.P. to cirrhotic rats for 1 week prior to measurement of platelet aggregation. The final injection of NAC/LA was given on the morning of analysis and 0.5mg/Kg LPS was administered between 2 and 4 hours post LA/NAC injection. Platelet aggregation was analysed after stimulation of PRP with 8μM ADP. This concentration of ADP was chosen as aggregation in BDL+LPS rats without NAC injection below 8μM was non-existent but 8μM ADP stimulated aggregation was also sub-maximal as 80μM ADP elicited significantly greater aggregation (Fig.18).

However, when the platelet aggregation was analysed after injection of NAC or LA there was no change in either the maximum aggregation or the aggregation profile (Fig.19, P=0.17). The non-significant trend was in fact a slight worsening of the platelet aggregation after injection of low molecular weight thiol antioxidants.

The observation that low molecular weight thiol antioxidants do not improve platelet dysfunction in endotoxaemic cirrhotic rats does not exclude a role for S-nitrosothiols in the dysfunction of platelet aggregation in liver disease, as they also failed to lower the plasma S-nitrosothiol concentration (see fig.16).

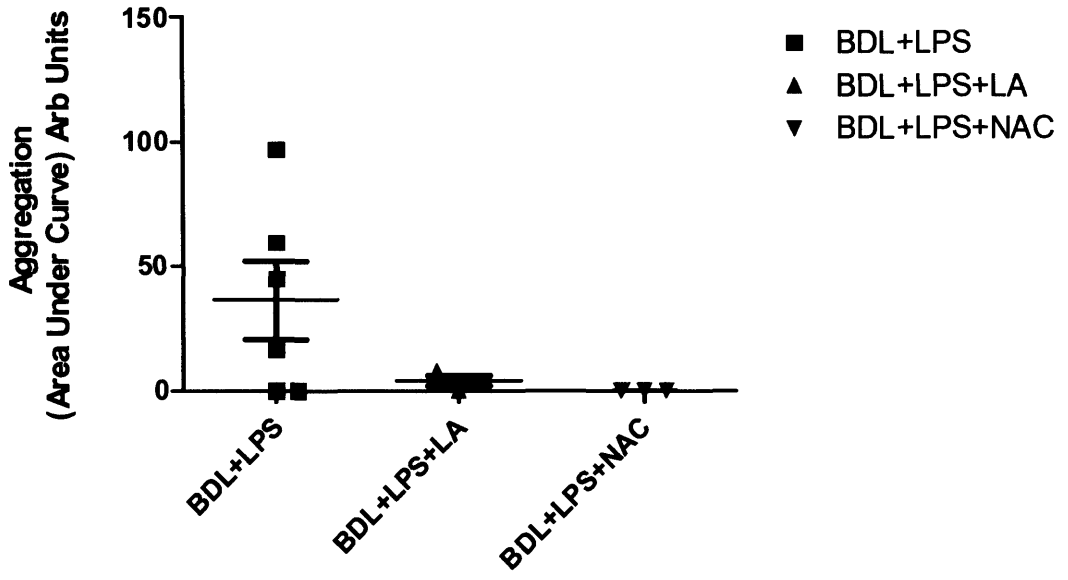


Figure 19. Effect of NAC and lipoic acid on platelet aggregation in endotoxaemic cirrhotic rats. Platelet aggregation in endotoxaemic cirrhotic rats was not improved by administration of the low molecular weight antioxidants N-acetylcysteine (NAC) or Lipoic acid (LA) in the 7 days prior to investigation of platelet aggregation. It was observed that injection of either NAC or LA injection seemed to lower the platelet aggregation in endotoxaemic cirrhotic rats, however this was not significant when analysed statistically (P=0.17). Statistis carried out by one-way ANOVA.

2.3.4. Mechanism of platelet dysfunction in endotoxaemia and cirrhosis

2.3.4.1. Nitric Oxide Synthase (NOS)

The incubation of platelets from endotoxaemic cirrhotic rats with L-NAME, a potent NOS inhibitor, had no effect on platelet aggregation (see Figure 20). Thus it would appear that the platelet dysfunction observed in endotoxaemic cirrhotic rats is independent of platelet NOS activity.

2.3.4.2. Soluble guanylate cyclase

When platelets isolated from endotoxaemic cirrhotic rats were incubated with ODQ *in vitro* there was no effect on platelet aggregation (see Figure 21). Thus, platelet dysfunction in endotoxaemic and cirrhotic rats is cGMP-independent. Whilst it is known that synthetically prepared S-nitrosothiols inhibit platelet aggregation when incubated with platelets *in vitro* through a predominantly cGMP-dependent mechanism, there is no data correlating high endogenous plasma S-nitrosothiol concentration and platelet dysfunction. S-Nitrosothiols are elevated to pharmacologically relevant concentration in endotoxaemic cirrhotic rats. Endogenous S-nitrosothiols must therefore inhibit platelet aggregation through a cGMP independent mechanism.

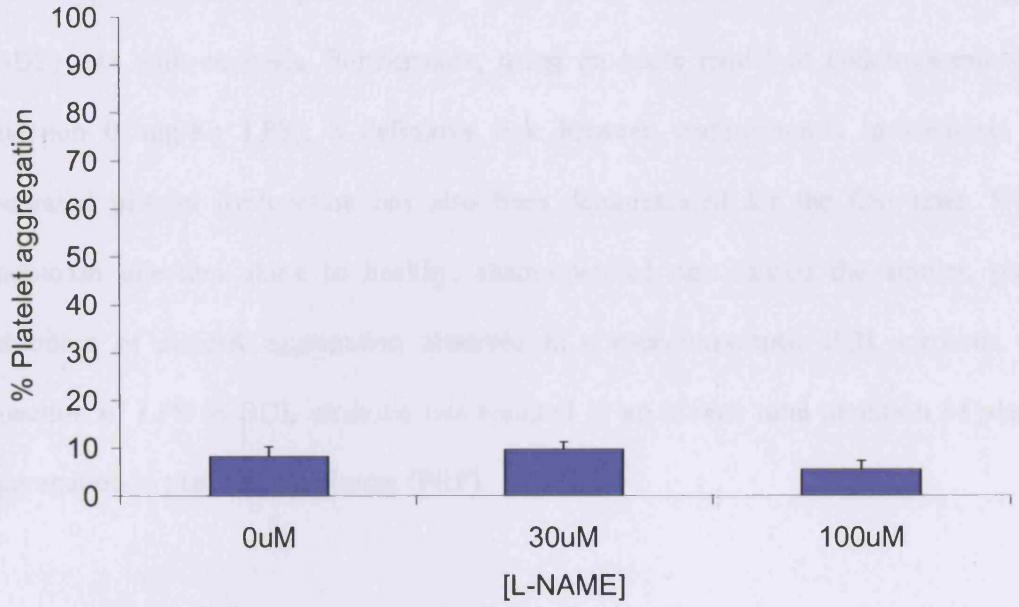


Figure 20. Incubation of platelets isolated from endotoxaemic cirrhotic rats with L-NAME had no effect on platelet aggregation. (n=5)

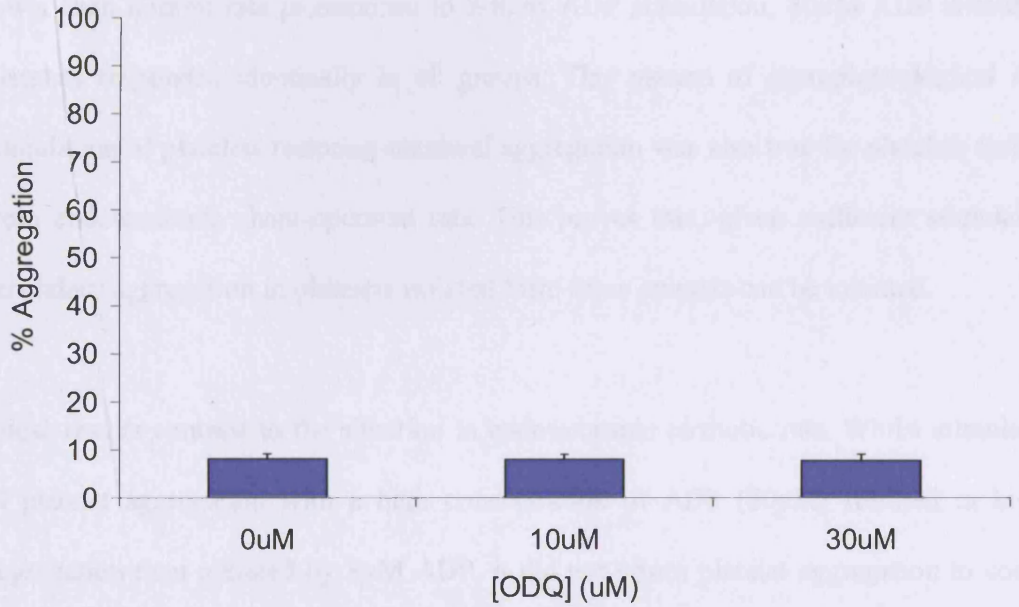


Figure 21. Incubation of platelets isolated from endotoxaemic cirrhotic rats with ODQ (guanylate cyclase inhibition) had no effect on platelet aggregation (n=4)

2.4. Discussion

This study establishes a platelet dysfunction in the chronic (25-day) bile duct ligated (BDL) rats with cirrhosis. Furthermore, using an acute model of endotoxaemia (2hr. injection 0.5mg/Kg LPS), a definitive link between endotoxaemia in cirrhosis and increased platelet dysfunction has also been demonstrated for the first time. Whilst endotoxin injection alone to healthy, sham-operated rats caused the similar, partial inhibition of platelet aggregation observed in non-endotoxaemic BDL cirrhotic rats, injection of LPS in BDL cirrhotic rats resulted in an almost total abolition of platelet aggregation in platelet rich plasma (PRP).

In sham-operated rats, maximum aggregation was observed at around 8μM ADP. However, whilst platelet aggregation recorded in cirrhotic rats rats was significantly lower than noraml rats in response to 2-8μM ADP stimulation, 80μM ADP stimulated platelets responded identically in all groups. This pattern of supraphysiological ADP stimulation of platelets restoring maximal aggregation was also true for platelets isolated from endotoxaemic sham-operated rats. This proves that, given sufficient stimulation, secondary aggregation in platelets isolated form these animals can be initiated.

These results contrast to the situation in endotoxaemic cirrhotic rats. Whilst stimulation of platelet aggregation with a high concentration of ADP (80μM) resulted in higher aggregation than initiated by 8μM ADP, it did not return platelet aggregation to control levels.

In order to investigate the mechanism of platelet dysfunction in endotoxaemia and/or cirrhosis, possible intrinsic and humoural mechanisms were examined. The role of intracellular NO generation by platelet NOS in endotoxaemic, cirrhotic rats was investigated using L-NAME, a NOS inhibitor, and was found not to affect platelet aggregation *in vitro* (Figure 20). Inhibition of soluble guanylate cyclase with ODQ likewise failed to improve platelet function in cirrhosis±endotoxaemia, ruling out a role for the L-arginine-NO-cGMP pathway by a humoral or intrinsic factor (Figure 21). Whilst NO production was increased in cirrhotic and/or endotoxaemic rats (as assessed by plasma nitrite+nitrate concentration), S-nitrosothiols were only detected in the plasma of endotoxaemic cirrhotic rats (BDL cirrhotic+LPS). Likewise, the concentration of the previously undifferentiated mercury-stable NO-carrying species (HgSNOCS) was only detected in significant concentration in endotoxaemic cirrhotic rats. It is therefore shown that whilst S-nitrosothiols may have a role in the dysfunction in platelet aggregation observed in endotoxaemic cirrhotic rats (through a cGMP-independent mechanism), they do not contribute to the platelet dysfunction observed in BDL cirrhotic or endotoxaemic rats.

The injection of the low molecular weight antioxidant, N-acetylcysteine (NAC), has previously been shown to lower S-nitrosothiol concentration when co-infused with S-nitrosoalbumin[250] and lipoic acid had previously been demonstrated to lower plasma S-nitrosothiol concentration in cirrhotic rats (unpublished data). NAC was therefore administered to endotoxaemic cirrhotic rats to investigate the effect of S-nitrosothiols on platelet aggregation. However, injection of NAC to endotoxaemic cirrhotic rats did not

result in a fall in endogenous plasma [S-nitrosothiol], but rather led to a fall in plasma [HgSNOCS]* (see Fig.16). Although the failure of NAC to lower plasma S-nitrosothiol concentration does not probe the effect of S-nitrosothiol concentration in platelet dysfunction in endotoxaemia and cirrhosis it may probe the role of HgSNOCS in this dysfunction. It was found that 7 day injection of NAC (or another low molecular weight thiol antioxidant, lipoic acid) did not affect platelet aggregation in endotoxaemic cirrhotic rats (See Figure 19). The high plasma concentration of HgSNOCS in endotoxaemic cirrhotic rats therefore appears not to be responsible for the inhibition of platelet aggregation observed. However, as injection of NAC only reduced the concentration of HgSNOCS in the plasma to 0.5μM, this cannot be totally discounted at this stage.

An interesting finding in this study was the observation that the total (protein and low molecular weight) plasma free thiol concentration (reduced sulphydryl concentration) is markedly decreased (by 75%) in BDL cirrhotic rats, but only 29% lower in endotoxaemic rats. This difference is probably due to the acute (2-hour) nature of the endotoxin challenge in this study and the chronic nature of the cirrhotic model (25 days) and therefore the relative exposure to conditions of oxidative stress. As low molecular weight thiols are only present in low micromolar concentration in the circulation, the distinct reduction in free plasma thiol concentration observed here presumably reflects a fall in protein free thiol concentration. This is the first study evaluating total (low molecular weight and protein) free thiol concentration in cirrhosis and the dramatic fall in plasma protein thiol concentration observed is therefore of potential pathological interest. Of

* As this was not differentiated in the unpublished study into lipoic acid from plasma [S-nitrosothiol] this explains the apparent fall in plasma S-nitrosothiol concentration following LA supplementation.

particular interest for further studies is the observation that the redox state of platelet surface protein "protein disulphide isomerase" (PDI) is important in the induction of platelet aggregation and that whole blood coagulation can be modulated by thiol redox status[245].

In conclusion, the induction of endotoxaemia in cirrhosis causes a profound impairment of platelet aggregation in cirrhotic rats, over and above the sum of dysfunction caused by induction of either cirrhosis or endotoxaemia alone. This correlates with the observation that the risk of gastrointestinal bleeding in cirrhotic patients is increased in cirrhotic those with bacterial infection. The gross dysfunction in aggregation in endotoxaemic cirrhotic rats also correlated with a gross elevation of plasma anti-platelet S-nitrosothiol concentration. However, the injection of the thiol antioxidant, NAC, which has been proposed to reduce plasma S-nitrosothiol concentration neither lowered S-nitrosothiol concentration nor improved platelet aggregation in endotoxaemic cirrhotic rats. Instead, it reduced the concentration of HgSNOCS, the concentration of which were also grossly elevated in the plasma of endotoxaemic cirrhotic rats. It therefore remains to be evaluated whether the dysfunction observed in endotoxaemic cirrhotic rats is also observed in infected cirrhotic patients and whether there is a role for S-nitrosothiols in this dysfunction.

Chapter 3: The role of S-nitrosoalbumin in platelet dysfunction in cirrhosis

3.1. Introduction

To characterise the role of S-nitrosoalbumin in platelet dysfunction in cirrhosis ± endotoxaemia, I employed a mutant strain of rats deficient in albumin. I also, for the first time, generated a model of cirrhosis in analbuminaemic rats so that the role of this pathway in cirrhosis could be investigated.

The discovery that injection of low molecular weight thiol antioxidants does not in fact decrease the concentration of plasma S-nitrosothiol meant it was not possible to use these compounds to probe the effect of S-nitrosothiols on platelet aggregation in endotoxaemic cirrhotic rats. An alternative method to evaluate the effect of lower plasma S-nitrosothiol concentration was therefore employed. An analbuminaemic strain of rats was obtained and the effect of induction of cirrhosis and/or endotoxaemia on platelet aggregation and plasma S-nitrosothiol concentration was determined. It is known that up to 95% of circulating S-nitrosothiols circulate in the form of S-nitrosoalbumin, the predominant plasma thiol, and therefore it was hypothesised that an analbuminaemic strain of rats will not be as susceptible to S-nitrosothiol formation. If plasma S-nitrosothiol concentration is important in dysfunctional platelet aggregation induced by endotoxaemia in cirrhosis, the analbuminaemic rat strain may be protected from dysfunctional platelet aggregation observed under these conditions.

Analbuminaemic (NAR) rats have previously been shown to respond to ADP-induced aggregation to the same extent as do normal Sprague Dawley (SpD) rats[241]. However, the response of NAR rats to induction of cirrhosis must be compared in order to assess any differences between platelet dysfunction in NAR and SpD strains. To this end, a model of BDL cirrhosis in analbuminaemic rats was established and characterised.

3.2. Materials and Methods

3.2.1. Analbuminaemic rats

Analbuminaemic rats were kept in identical conditions to Sprague Dawley rats as described in Chapter 2. They were sourced and bred from a colony established at the Comparative Biology Unit at the Royal Free Hospital, Royal Free and University College Medical School.

3.2.2. Analbuminaemic model of cirrhosis

A model of cirrhosis in analbuminaemic (NAR) rats was established by bile duct ligation as described in chapter 2. The progression of liver disease following ligation was monitored by liver histology and plasma biochemistry and these were compared to the progression of disease in normal Sprague Dawley rats.

3.2.3. Other Materials and Methods

All other materials and methods used were identical to those described in chapter 2.

3.3.Results

3.3.1. Establishment of a model of biliary cirrhosis in the analbuminaemic rat and comparison with the Sprague Dawley rat following bile duct ligation

3.3.1.1. Histology

There was no histological difference in the progression of liver disease in analbuminaemic (NAR) and Sprague Dawley (SpD) rats following bile duct ligation. One (1 out of 3) analbuminaemic cirrhotic+LPS rat had an Ishak score of 3 (Fibrous expansion of most portal areas, with occasional portal to portal bridging), but otherwise the results were consistently an Ishak score of 5 (Marked bridging (portal to portal and portal-central) with occasional nodules (incomplete cirrhosis). All Sprague Dawley cirrhotic rat livers were also scored at an Ishak score of 5.

3.3.1.2. Biochemical parameters of liver disease

Plasma AST activity increased following bile duct ligation in both Sprague Dawley (SpD) and analbuminaemic (NAR) rats (see Figure 22). Injection of LPS to cirrhotic rats increased AST activity in both strains but this rise was not statistically significant in either strain. Plasma AST activity in sham NAR rats was slightly higher than in sham SpD rats but this was not statistically significant. The increase in plasma AST activity

following bile duct ligation was the same in SpD rats as NAR rats.(Figure 23). Whilst bile duct ligation increased plasma ALT activity in SpD rats there was no increase in NAR rats. However, there was no significant difference in the absolute ALT levels in SpD and NAR rats following bile duct ligation. The lack of a rise in ALT concentration following bile duct ligation in NAR rats may represent a different pathology following bile duct ligation but this is not supported by histology. Another explanation could be that the increased plasma ALT activity in NAR rats indicates an underlying liver dysfunction which bile duct ligation does not further exacerbate. There is no histological evidence for this either. ALT is not confined to the liver so in the absence of histological evidence of liver disease it is probable that the analbuminaemic rat is releasing ALT from another tissue.

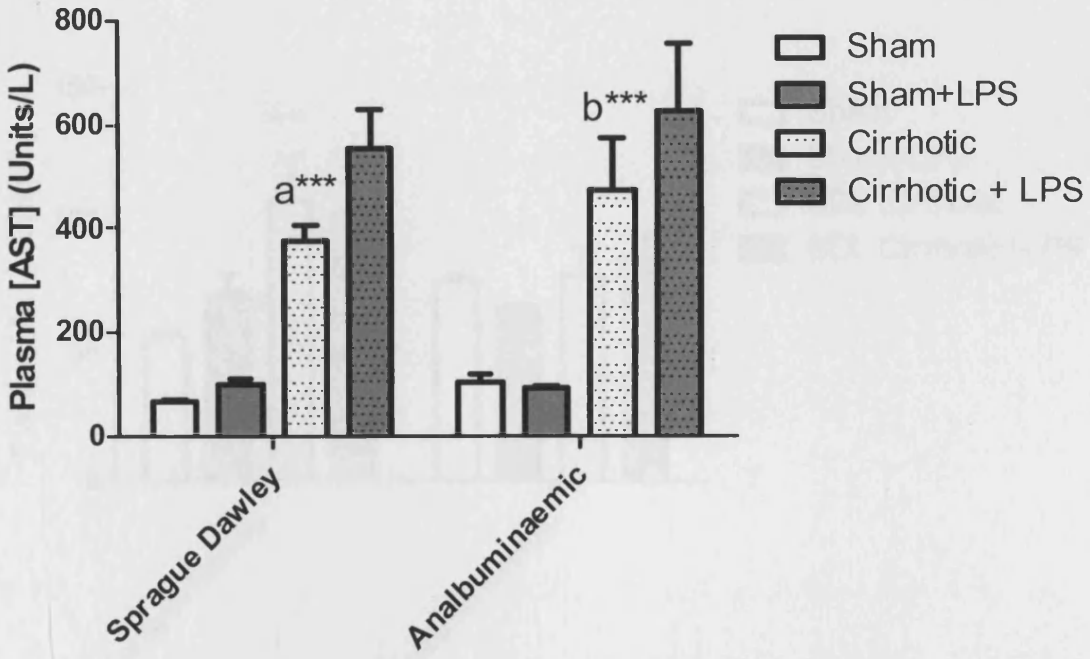


Figure 22. Plasma AST concentration in normal and cirrhotic Sprague Dawley and Analbuminaemic rats ± endotoxaemia. There was no difference in [AST] between Sprague Dawley and analbuminaemic strains in any group (P=0.23), but there was a difference between groups (P<0.0001). Statistic carried out by Two-Way ANOVA with Newman-Keuls post-hoc comparisons. A = vs. Sham Sprague Dawley rats, b = vs. Sham analbuminaemic rats. ***p<0.001. n ≥ 5 in all groups

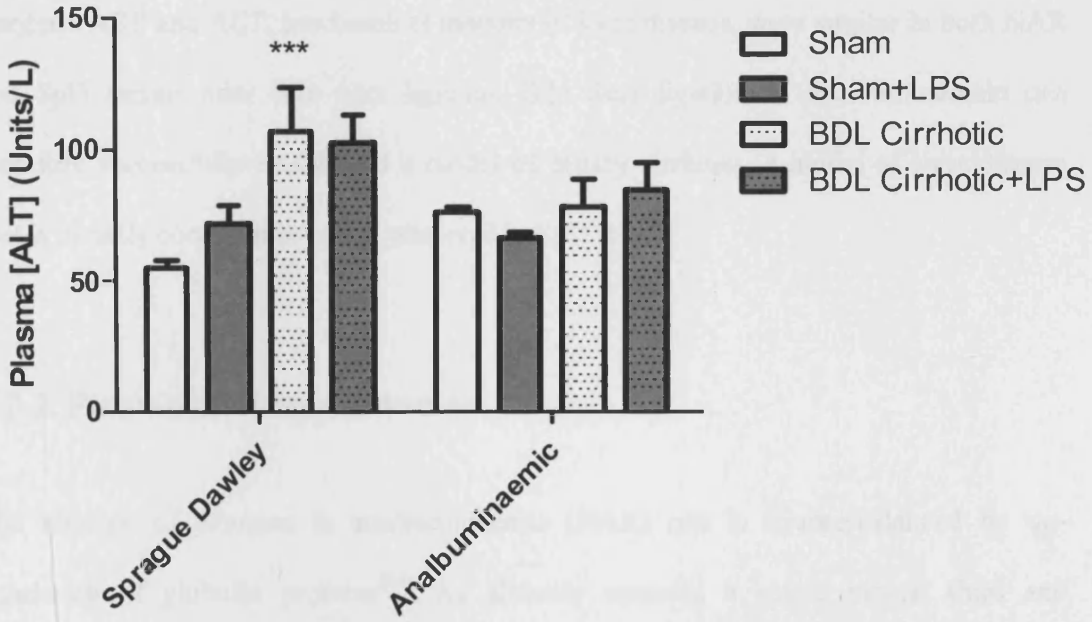


Figure 23. Plasma [ALT] activity in normal and cirrhotic Sprague Dawley and analbuminaemic rats ± endotoxaemia. The pattern of plasma [ALT] in Sprague Dawley and Analbuminaemic strains was different as analysed by two-way ANOVA (P=0.0221). In Sprague Dawley cirrhotic rats there was an elevation in plasma [ALT] above sham operated rats ($p<0.001$). However, induction of cirrhosis had no effect on [ALT] in analbuminaemic rats. Statistical analysis carried out by two-way ANOVA with Newman-Keuls post-hoc comparisons. ***$p<0.001$ vs. sham operated Sprague Dawley rats.

3.3.1.3. Conclusions

Histological changes in the liver following bile duct ligation were identical in analbuminaemic (NAR) rats and Sprague Dawley rats. The plasma activities of the liver enzymes AST and ALT, biochemical markers of liver disease, were similar in both NAR and SpD strains after bile duct ligation. Bile duct ligation in analbuminaemic rats therefore successfully established a model of biliary cirrhosis, a model of liver disease that is directly comparable to that observed in SpD rats.

3.3.2. Plasma thiol concentration

The absence of albumin in analbuminaemic (NAR) rats is counterbalanced by up-regulation of globulin proteins[234]. As albumin contains a single vicinal thiol and represents the majority of the thiol pool in plasma, it was expected that NAR rats would have a dramatically lower plasma thiol concentration than normal (Sprague Dawley, SpD) rats. Plasma free thiols (reduced sulphydryl) were indeed significantly lower in sham operated NAR rats than SpD rats with or without endotoxaemia (238±13μM SpD sham, 27±4μM NAR sham and 169±20μM SpD sham+LPS, 32±4μM NAR sham+LPS, $P<0.0001$, two-way ANOVA). However, the free thiol concentration in cirrhotic NAR rats was increased compared to sham NAR rats (27±4μM sham NAR vs. 64±5μM BDL cirrhotic NAR). This may represent the effects of an up-regulation of antioxidants, particularly glutathione in cirrhosis. The concentration of thiols in Sprague Dawley and

NAR BDL cirrhotic rats was very similar (SpD cirrhotic 60±7μM, NAR cirrhotic 64±5μM). Injection of LPS to NAR cirrhotic rats lowered the plasma thiol concentration to the same degree as observed in SpD rats (64±5μM NAR cirrhotic, 27±4μM NAR cirrhotic +LPS).

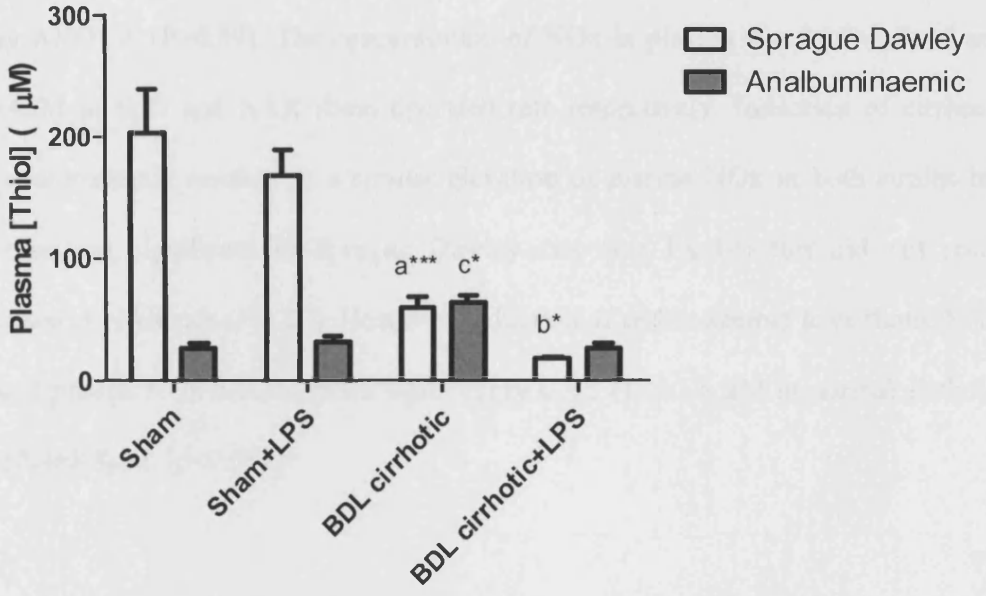


Figure 24. Plasma thiol concentration in normal and cirrhotic Sprague Dawley (SpD) and analbuminaemic (NAR) ± endotoxaemia. Plasma thiol concentration in NAR rats is significantly different to SpD rats presumably due to the absence of albumin ($P<0.0001$). Induction of cirrhosis in SpD animals resulted in a dramatic fall in free thiol concentration ($p<0.001$), which was exacerbated following acute injectio of LPS ($p<0.05$). Thiol concentration is slightly higher in the plasma of BDL cirrhotic NAR rats than Sham controls perhaps due to upregulation of thiol antioxidants such as glutathione ($p<0.05$). Statistics performed using two-way ANOVA with Newman-Keuls post-hoc comparisons. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. $n \geq 5$ in all groups. a = vs. SpD sham, b = vs. Spd BDL, c = vs. NAR sham,

3.3.4. Plasma nitrite + nitrate concentration

There was no significant difference between the plasma nitrite + nitrate (NOx) concentration in Sprague Dawley (SpD) and analbuminaemic (NAR) rats as assessed by two-way ANOVA (P=0.89). The concentration of NOx in plasma was 14.9±1.3μM and 14.7±0.4μM in SpD and NAR sham operated rats respectively. Induction of cirrhosis and/or endotoxaemia resulted in a similar elevation of plasma NOx in both strains but whilst this was significant in Sprague Dawley rats (see Fig.14) this did not reach significance in NAR rats (Fig.27). However, induction of endotoxaemia in cirrhotic NAR rats raised plasma NOx concentration significanty from 21.9±1.6 μM in normal cirrhotic rats to 50.4±8.8μM, ($p<0.001$).

The observation that NO synthesis in SpD and NAR rats is similar under both physiological and pathological conditions implies that the absence of albumin in NAR rats does not affect the synthesis of NO in these rats.

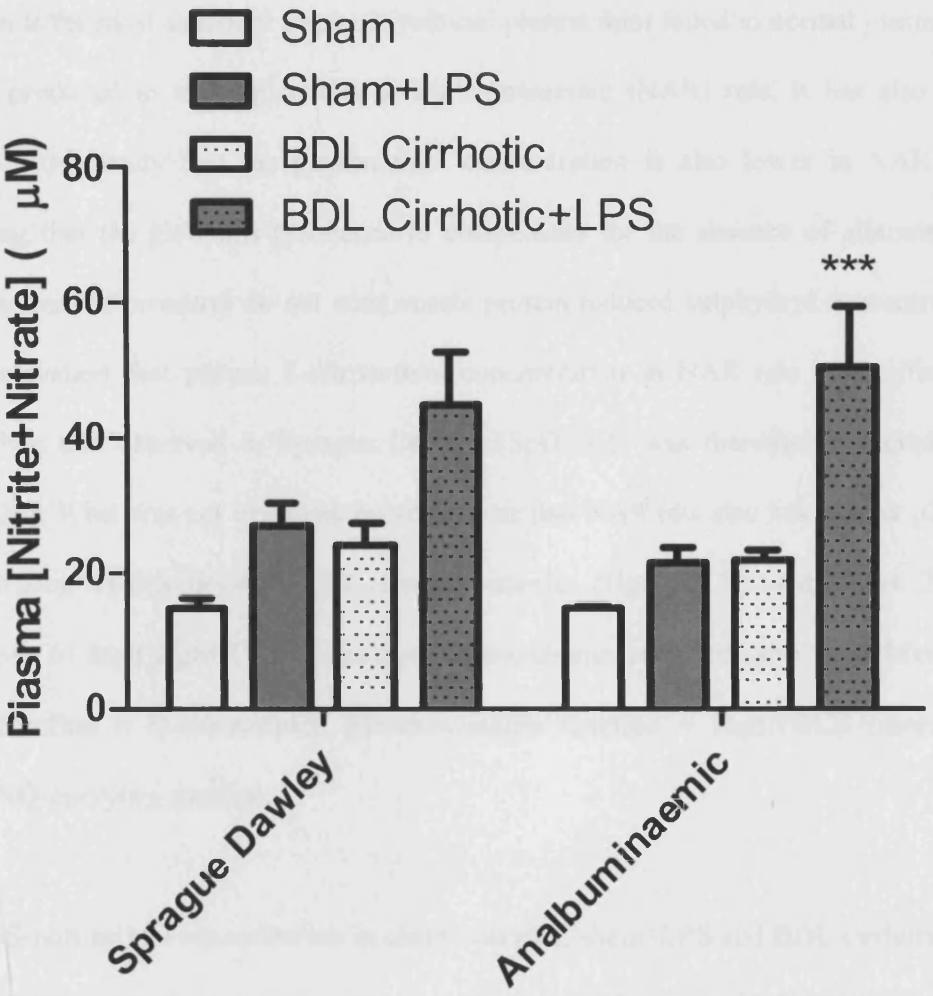


Figure 25. Plasma Nitrite + Nitrate concentration in the plasma of normal and cirrhotic SpD and NAR rats ± endotoxaemia. There was no difference in the pattern of plasma nitrite + nitrate (NOx) concentration elevation following induction of cirrhosis±endotoxaemia in Sprague Dawley (SpD) and analbuminaemic rats (NAR) (P=0.89). The increase in [NOx] after induction of endotozxaemia or cirrhosis was not significant in NAR rats, however, the increase in plasma [NOx] following injection of LPS to BDL cirrhotic NAR rats was highly significant ($p<0.001$). Statistics performed by two-way ANOVA with Newman-Keuls post-hoc comparisons on Analbuminaemic group. For post-hoc testing of Sprague Dawley rats see Fig.14. ***$p<0.001$ vs. NAR BDL. $n \geq 8$ in all SpD groups and $n \geq 5$ in analbuminaemic rats.

3.3.5. Plasma S-nitrosothiols and HgSNOCS

Albumin is the most abundant source of reduced plasma thiol found in normal plasma but is only produced in trace quantities in analbuminaemic (NAR) rats. It has also been shown in this study that the plasma thiol concentration is also lower in NAR rats, indicating that the globulins (produced to compensate for the absence of albumin and maintain oncotic pressure) do not compensate protein reduced sulphydryl concentration. The observation that plasma S-nitrosothiol concentration in NAR rats is significantly lower than that observed in Sprague Dawley (SpD) rats was therefore expected (see Figure 26). What was not expected, however, was that NAR rats also have lower plasma concentration of mercury-stable NO-carrying species (HgSNOCS) (see Figure 27) in conditions of high HgSNOCS formation (endotoxaemia and cirrhosis). **NB. Mercury labile fraction = S-nitrosothiol, Mercury-stable fraction = HgSNOCS (mercury-stable NO-carrying species).**

Plasma S-nitrosothiol concentration in sham-operated, sham+LPS and BDL cirrhotic rats of both NAR and SpD strains were all below the limit of detection. HgSNOCS were also below the detection limit of the assay in sham-operated and sham+LPS rats. HgSNOCS in BDL cirrhotic rats were very low: 13±3nM and 21.5±14nM in SpD and NAR rats respectively. As previously noted, injection of LPS to induce endotoxaemia in cirrhotic Sprague Dawley rats caused a large rise in plasma S-nitrosothiol concentration (13±3nM sham vs. 659±209nM BDL+LPS). As predicted, injection of LPS to NAR BDL cirrhotic rats did not lead to such a large rise in plasma S-nitrosothiol concentration. Plasma S-

nitrosothiol concentration was 98±54nM, $p<0.05$ vs. SpD BDL+LPS rats (659±209nM). What was also interesting and unexpected was the observation that the plasma HgSNOCS concentration also increased markedly in endotoxaemic cirrhotic rats of both strains but was also lower in NAR endotoxaemic cirrhotic rats than SpD endotoxaemic cirrhotic rats (1941±557nM SpD vs. 521±198nM NAR, $p<0.05$). This suggests the possibility that albumin is involved directly or indirectly in the formation of HgSNOCS, possibly through protein thiols or stabilisation of nitrosonium anion in the hydrophobic core.

The reason for this difference in the concentration of plasma S-nitrosothiols is presumably the lower thiol and/or albumin concentration in NAR rats. The reason for the difference in HgSNOCS concentration in NAR rats is less easily explained. There is no difference between the concentration of plasma NOx produced in SpD and NAR strains. The exposure of NAR and SpD rat plasma to NO is therefore presumably the same and therefore this indicates that the formation of HgSNOCS is either albumin concentration dependant or NO independent. Albumin may catalyse the formation of HgSNOCS in SpD plasma through stabilisation of nitrosating species such as N_2O_3 or S-nitrosoalbumin may itself form HgSNOCS in a transnitrosation reaction. It is also possible that the predominant HgSNOCS is albumin associated. Alternatively, a molecule such as peroxynitrite may be responsible for HgSNOCS formation and may be produced in different quantities in SpD and NAR rats.

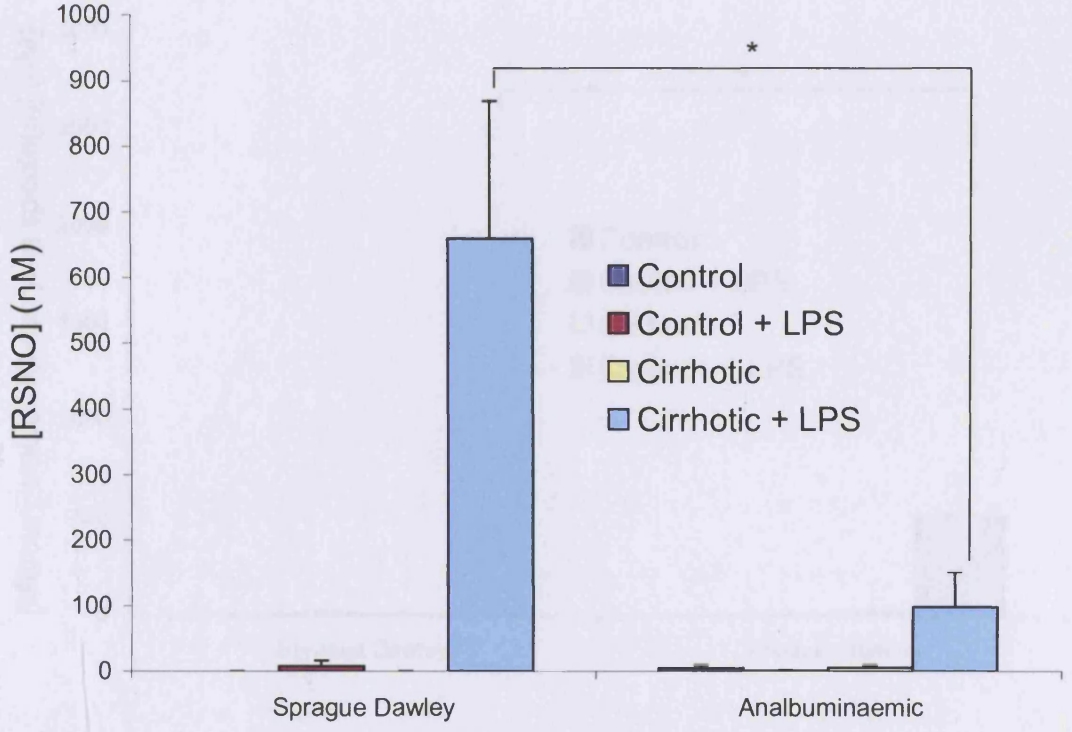


Figure 26. Plasma S-nitrosothiol (RSNO) concentration in normal and cirrhotic Sprague Dawley and analbuminaemic rats ± endotoxaemia. Consistent with lower reduced plasma thiol concentration, endotoxaemic cirrhotic analbuminaemic rats develop lower S-nitrosothiol concentration than Sprague Dawley rats. The difference between plsma [S-nitrosothiol] in Sprague Dawley and analbuminaemic endotoxaemic cirrhotic rats was analysed by unpaired, two-tailed t-test ($p=0.024$) ignoring the other data sets as these were at the limit of detection and therefore discounted for the purposes of statistical analysis. $n \geq 3$ for all groups except n=7 for cirrhotic+LPS rats.

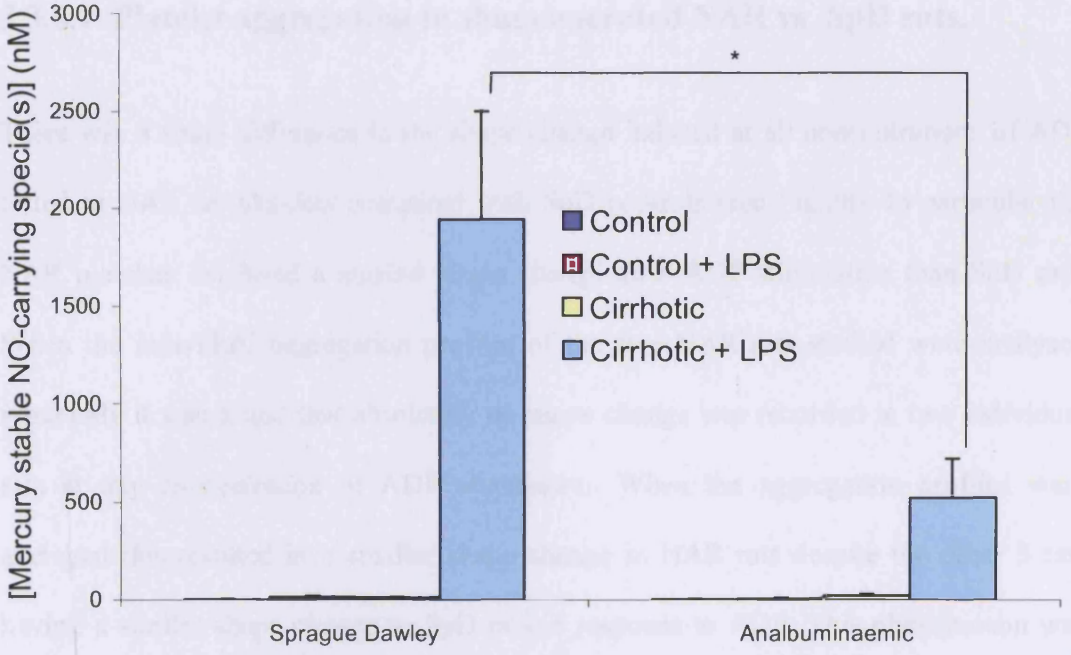


Figure 27. Plasma mercury-stable NO-carrying species (HgSNOCS) concentration in normal and cirrhotic Sprague Dawley and analbuminaemic rats ± endotoxaemia. Endotoxaemic cirrhotic analbuminaemic rats also have lower HgSNOCS concentration than do Sprague Dawley rats. The difference between plsma [HgSNOCS] in Sprague Dawley and analbuminaemic endotoxaemic cirrhotic rats was analysed by unpaired, two-tailed t-test ($p=0.033$) ignoring the other data sets as these were at the limit of detection and therefore discounted for the purposes of statistical analysis. * = $p<0.05$. $n \geq 3$ for all groups except n=7 for cirrhotic+LPS rats.

3.3.6. Platelet aggregation in Sprague Dawley and NAR rats

3.3.6.1. Platelet aggregation in sham operated NAR vs. SpD rats.

There was a small difference in the shape change induced at all concentrations of ADP tested in NAR rat platelets compared with SpD controls (see Fig.28). In particular the NAR platelets exhibited a smaller shape change after ADP stimulation than SpD rats. When the individual aggregation profiles of the five NAR rats studied were analysed separately it was found that absolutely no shape change was recorded in two individual rats at any concentration of ADP stimulation. When the aggregation profiles were averaged this resulted in a smaller shape change in NAR rats despite the other 3 rats having a similar shape change to SpD rats in response to ADP. This phenomenon was noted seemingly at random in the aggregation profile of various NAR rats with or without cirrhosis and/or LPS injection. Whilst the platelets isolated from some NAR rats underwent normal shape changes others would seemingly undergo no shape change. Occasionally, platelets from SpD rats exhibit an impaired shape change but far less regularly than in NAR rats. There was a small but statistically significant decrease in platelet aggregation at low concentrations of ADP (0.8μM), but this difference was absent at higher concentrations of ADP.

The difference between platelet aggregation in 2μM ADP stimulated SpD and NAR platelets was not statistically significant at any time point. Three out of five NAR rats

showed a sharp disaggregation after maximum aggregation was achieved whilst the other 2 maintained maximum aggregation for the duration of the recording time. In comparison, only one out of 4 SpD platelet aggregation profiles sharply disaggregated after maximum aggregation was achieved whilst 3 maintained maximum aggregation. There may therefore be a difference in the disaggregation profile of NAR and SpD rats at sub maximal ADP concentration but the aggregation of platelets appears to be identical in SpD and NAR rats at 0.8-80μM ADP stimulation concentrations.

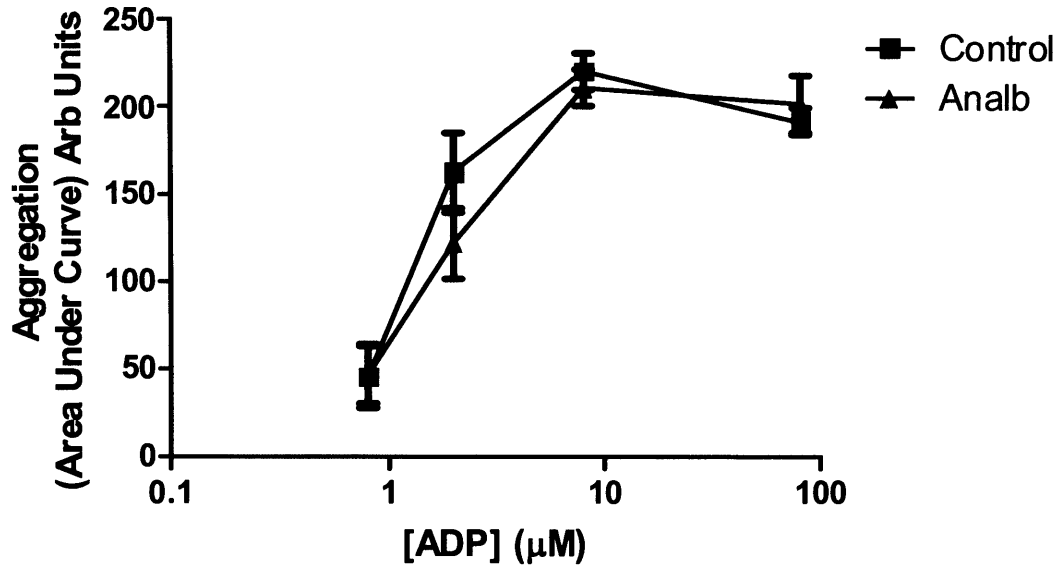


Figure 28. Platelet aggregation profile in sham operated Sprague Dawley (SpD) and analbuminaemic (NAR) rats induced by increasing concentrations of ADP As has previously been shown[240], The platelet aggregation profile of SpD and NAR rats is identical at all concentrations of ADP tested as assessed by two-way ANOVA (P=0.24). n=6 SpD, n=5 NAR

3.3.6.2. Effect of endotoxaemia on platelet aggregation

Induction of endotoxaemia by injection of 0.5mg/Kg LPS, 2hr in NAR rats resulted in no significant dysfunction in platelet aggregation at any ADP stimulation concentration unlike in Sprague Dawley rats, (see Fig.29). The aggregation in NAR endotoxaemic rats was significantly greater than that observed in endotoxaemic SpD rats at 0.8 and 2μM ADP stimulation concentrations. Analbuminaemic rats are therefore protected against the platelet dysfunction induced in Sprague Dawley rats.

3.3.6.3. Effect of induction of cirrhosis

Platelets isolated from SpD cirrhotic rats exhibit impaired aggregation compared with sham operated rats (see Fig.30). Likewise platelets isolated from NAR cirrhotic rats also display slightly impaired platelet aggregation compared to sham controls at 0.8-8μM ADP stimulation, however, this did not reach statistical significnace. There was no difference between the dysfunction in platelet aggregation induced by cirrhosis in SpD and NAR rats at any ADP stimulation concentration.

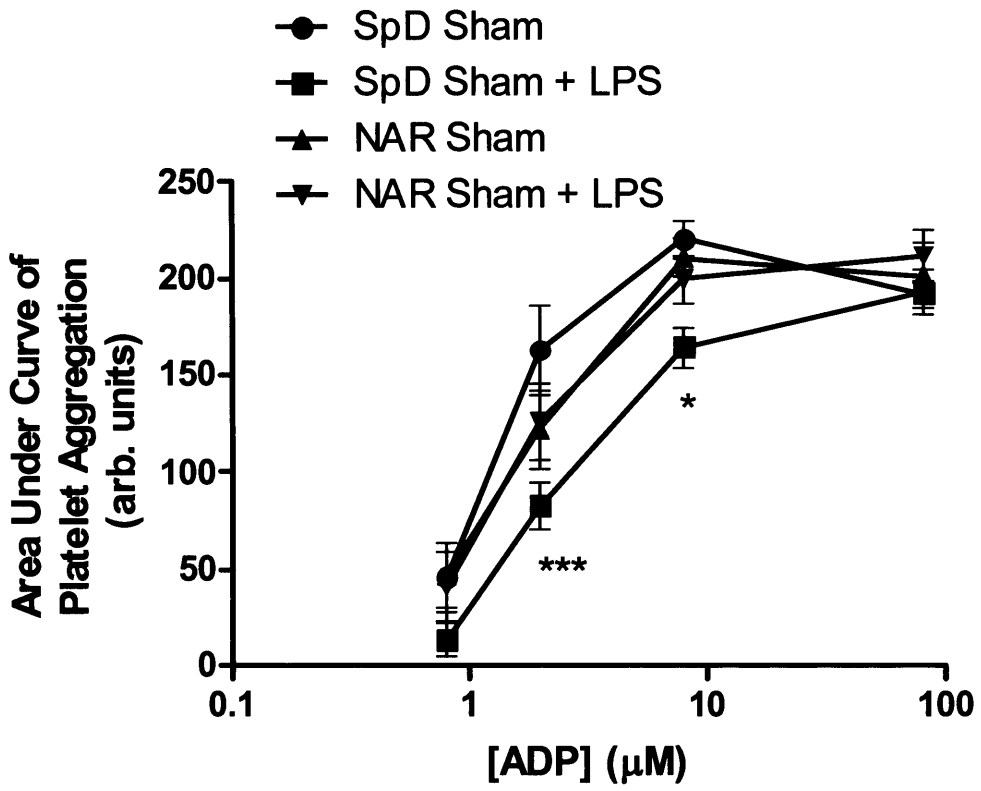


Figure 29. Platelet aggregation in endotoxaemic sham operated Sprague Dawley (SpD) and analbuminaemic (NAR) rats induced by increasing concentrations of ADP. Platelets from analbuminaemic rats showed no impairment of aggregation following injection of LPS (statistics performed using two-way ANOVA with Newman-Keuls post-hoc comparisons: $p>0.05$ between NAR sham and NAR sham + LPS at all ADP concentrations). This contrasted to the significant impairment of platelet aggregation at 2μM and 8μM [ADP] induced LPS injection in SpD rats. * $p<0.05$, ***$p<0.001$ vs. SpD Sham. n=6 SpD ± LPS, n=5 NAR ± LPS.

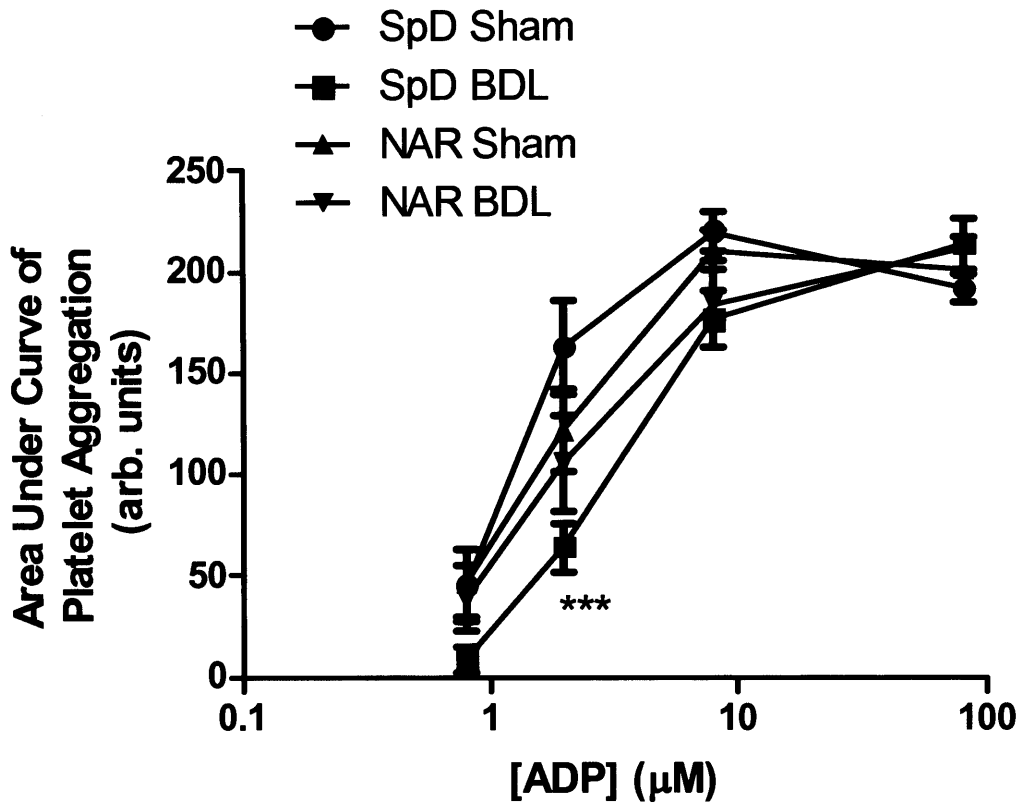


Figure 30. Platelet aggregation in cirrhotic Sprague Dawley (SpD) and analbuminaemic (NAR) rats induced by increasing concentrations of ADP. Platelets from analbuminaemic rats showed no impairment of aggregation following injection of cirrhosis (statistics performed using two-way ANOVA with Newman-Keuls post-hoc comparisons, $p>0.05$ between NAR sham and NAR sham + LPS at all ADP concentrations). This contrasted to a significantly reduced platelet aggregation in SpD cirrhotic rats compared to SpD sham operated rats upon stimulation with 2μM ADP. ***$p>0.001$ vs. SpD Sham. n=6 SpD Sham, n=5 NAR sham. n=7 SpD BDL, n=6 NAR BDL.

3.3.6.4 Effect of induction of endotoxaemia in cirrhosis

Injection of LPS to cirrhotic Sprague Dawley rats resulted in a marked impairment of platelet aggregation upon 2-80μM ADP stimulation when compared to non-endotoxaemic cirrhotic rats (see Fig.31). When cirrhotic analbuminaemic rats were injected with the same dose of LPS, the platelet aggregation measured was also significantly lower than that observed in non-endotoxaemic cirrhotic rats at 2μM ADP stimulation (Fig. 31). More important though was the observation that the dysfunction of platelet aggregation observed in endotoxaemic cirrhotic NAR rats was significantly less than observed in endotoxaemic cirrhotic SpD rats. This trend can be observed at 2-80μM ADP stimulated aggregation concentrations and is highly significant in 8μM ADP stimulated platelets. Analbuminaemic rats therefore appear to be partially protected against dysfunction of platelet aggregation induced by endotoxaemia in cirrhosis, as was hypothesised.

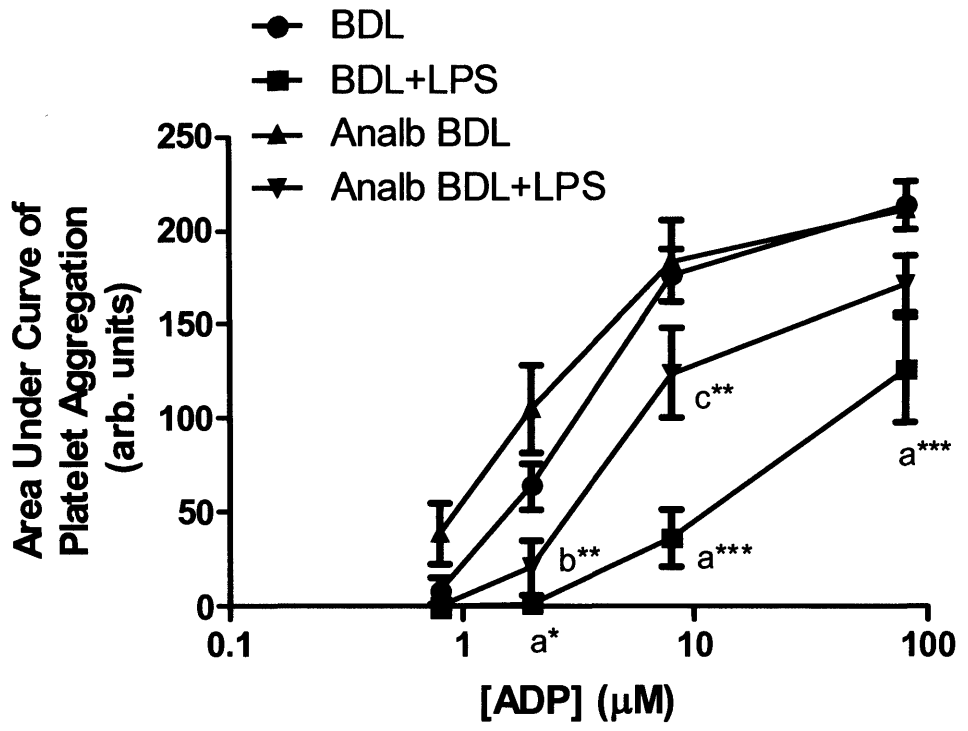


Figure 31. Platelet aggregation profile in endotoxaemic cirrhotic Sprague Dawley (SpD) and analbuminaemic (NAR) rats induced by increasing concentrations of ADP. Platelets from analbuminaemic rats showed a lessened impairment of aggregation following injection LPS to cirrhotic rats than the equivalent SpD rats (statistics performed using two-way ANOVA with Newman-Keuls post-hoc comparisons, $p>0.05$ between NAR sham and NAR sham + LPS at all ADP concentrations bar 2μM. At this concentration LPS injection reduced platelet aggregation, $p<0.01$). Platelets isolated from endotoxaemic cirrhotic SpD rats displayed impaired aggregation compared to non-infected controls at2-80μM concentration (for statistics see Fig.19) Significantly, platelets isolated from endotoxaemic cirrhotic NAR rats aggregated significantly more than those isolated from endotozxaemic cirrhotic SpD rats at 8μM concentration, indicating a protection from platelet dysfunction under these conditions in the NAR rat ($p<0.01$). *$p<0.05$, **$p<0.01$, ***$p<0.001$. a = vs. uninfected SpD cirrhotics, b = vs. uninfected NAR cirrhotics, c = vs. endotoxaemic cirrhotic SpD. n=7 SpD BDL, n=6 NAR BDL. n=5 SpD and NAR BDL+LPS.

3.3.7. Effect of NAC supplementation on analbuminaemic rats

As part of the continuing programme of investigation of low molecular weight thiol antioxidants, NAC was injected to cirrhotic analbuminaemic (NAR) rats parallel to the experiments in Sprague Dawley rats (see section 2.3.4.). NAC (100mg/Kg/day) was injected to 3 cirrhotic NAR rats and resulted in all 3 animals suffering severe, systemic haemorrhage manifested externally in the nails, eyes and nose. These symptoms were discovered in the morning, 15 hours after the first injection of NAC. One animal had died and the other two were put to sleep immediately on discovery of symptoms and the experiment terminated. On autopsy, it was discovered that there was internal haemorrhage and necrosis. These observations underline the importance of thiol redox balance in haemostasis. It is possible that the absence of albumin in NAR rats and the associated fall in protein thiol means that the NAC injected did not form mixed disulphides with albumin. NAC therefore became toxic as it disturbed thiol redox balance of key coagulation factors and perhaps platelet receptors resulting in the severe haemorrhage reported.

3.4. Discussion

Induction of cirrhosis by bile duct ligation in analbuminaemic rats resulted in the same histological pattern of fibrosis and resulted in similar elevation of transaminase enzymes in the plasma as found in Sprague Dawley (SpD) rats. Thus, for the first time a model of cirrhosis has been established in an analbuminaemic rat strain.

Analbuminaemic (NAR) rats have similar basal NO to Sprague Dawley (SpD) rats production as measured by nitrite+nitrate (NOx) accumulation in the plasma (Figure 25). Induction of endotoxaemia and/or cirrhosis also leads to similar increase in plasma NOx concentration, indicating that NAR and SpD rats induce NO production to the same extent under pro-inflammatory pathological conditions. However, the plasma thiol concentration in NAR rats is far lower than that in SpD under normal physiological conditions due to the absence of albumin (Fig.26). However, endotoxaemic cirrhotic NAR rats have similar thiol concentration to endotoxaemic cirrhotic SpD rats indicating that the lower S-nitrosothiol concentration observed in endotoxaemic cirrhotic NAR rats (Fig.28) is specifically the result of decreased plasma albumin and not simply available thiol concentration. This supports studies implicating albumin in the formation of S-nitrosothiols, possibly through the stabilisation of unstable nitrosating molecules in the albumin hydrophobic core. The observation that NAR rats have decreased concentration of mercury-stable NO-carrying species (HgSNOCS) was not expected and may signify a role of albumin as either the target for HgSNOCS formation or as a catalyst for HgSNOCS formation (Fig.29). Alternatively, HgSNOCS may be formed from a species

such as peroxynitrite and a differential superoxide dismutase activity may account for the difference in HgSNOCS concentration. This difference is revisited in a later chapter. Whilst these results suggest that HgSNOCS

The protection of NAR rats against platelet dysfunction in endotoxaemic cirrhotic rats compared to SpD rats and the lower S-nitrosothiol concentration in this strain is a compelling correlation (Fig.28 and Fig.33). Whilst not an absolute proof of the effect of S-nitrosothiols on platelet dysfunction in endotoxaemia and cirrhosis, the observation in two separate rat strains that the concentration of S-nitrosothiols, known platelet inhibitor molecules, corresponds to the degree of platelet dysfunction strongly implies a relationship. This is further supported by the observation that the dysfunction in platelet aggregation induced by cirrhosis or endotoxaemia separately does not lead to formation of significant S-nitrosothiol formation or such a degree of dysfunction of platelet aggregation (Fig.31 and Fig.32). There are probably other mechanisms involved in the protection from platelet dysfunction in analbuminaemic rats, notably thiol redox status as evidenced by the toxicity of NAC in NAR rats.

The observation that HgSNOCS concentration also corresponds to the degree of platelet dysfunction in endotoxaemia and cirrhosis again raises the question as to whether these molecules themselves may have biological activity.

Chapter 4: Identification of Mercury-stable NO-carrying species and evaluation of the biological activity

4.1. Introduction

The prevalence of mercury-stable NO-carrying species (HgSNOCS) in the plasma of endotoxaemic cirrhotic rats warranted further investigation. In order to generate sufficient quantities of HgSNOCS for analysis a method was successfully set up to generate these species *in vitro* using human plasma. Firstly the molecular weight of HgSNOCS formed were analysed by molecular weight cut off filters and by comparison to the fractionation profile of S-nitrosated albumin. The nature of the HgSNOCS was then investigated by assessing their stability to a range of chemicals known to decompose known NO-carrying molecules. Finally the anti-platelet activity was assessed and compared to that elicited by S-nitrosothiol.

4.2. Materials and methods

4.2.1. Preparation of mercury-stable NO-carrying plasma

In order to establish the properties and identity of the mercury-stable NO carrying species (HgSNOCS) in plasma it was necessary to obtain the product in large quantities. As a 300g rat has an approximate circulating blood volume of 20mL, of which it is possible to extract about 50%, it is only possible to obtain 5mL plasma per endotoxaemic cirrhotic rat. This is insufficient for many protocols and plasma isolated from endotoxaemic cirrhotic rats contains S-nitrosothiols as well as HgSNOCS. Human plasma was therefore incubated with 10mM final concentration of N-ethylmaleimide (NEM) to block the thiol groups and was then incubated with 1mM DETA-NONOate for 4 hours at 37°C under constant rotation. After incubation, plasma was dialysed against 3×3L of PBS + 100μM DTPA over 48 hours to remove excess NEM and DETA-NONOate. The plasma was then analysed for S-nitrosothiols, mercury-stable NO-carrying species (HgSNOCS), low molecular weight NO-carrying species and nitrite using the techniques described in earlier chapters.

In a set of experiments an identical protocol was followed, except that NEM was not added to the plasma. This control experiment was designed to ensure that any observed mercury-stable nitrosation was not simply an associated of NO with NEM.

4.2.2. Identification of mercury-stable NO-carrying species molecular weight

4.2.2.1. Molecular weight cut off filters

A 500μL 50:50 HgSNOCS/S-nitrosoalbumin solution was prepared. Each 500μL aliquot was then added to either a 30KDa or 100KDa (Millipore, Bedford, MA) molecular weight cut off filter and centrifuged at 10 000×g for 1hr. at which time all liquid had passed through the filter. The filtrate was analysed for S-nitrosothiol and HgSNOCS concentration. Further, the pellets from the filters were resuspended in PBS/NEM/DTPA and analysed for S-nitrosothiol and X-NO.

4.2.2.2. Size exclusion column

Sephadex G-25 columns (Amersham Pharma. Biotech, UK) were used to assess the size of the mercury-stable fraction in plasma. Columns were first washed extensively with PBS + 100μM DTPA + 10mM NEM. 800μL of a 4μM MS-NO-CP preparation was added to 800μL of a 4μM solution of S-nitrosoalbumin (synthesised by the Cys-NO transnitrosation method and diluted to 4μM in PBS/NEM/DTPA), resulting in a 50% mercury stable and 50% mercury labile solution. 400μL aliquots of this solution were then made up to 2.5mL in PBS/NEM/DTPA and applied to the Sephadex G-25 column. When all the solution had been absorbed, sequential 0.5mL aliquots of PBS/NEM/DTPA were applied to the column and the eluate collected in separate tubes. 2×200μL of the eluate was analysed on the NOA one with and one without $HgCl_2$ incubation.

4.2.3. Synthesis of S-nitrosoalbumin

Human serum albumin (HSA) was initially dissolved in PBS + 100μM DTPA at a concentration of 20mg/mL whilst stirring gently to avoid denaturation of protein. The thiol groups in the molecule were then reduced using 0.5mM dithiothreitol (DTT) in the dark for 2hr. and then dialysed against 3×3L PBS+100μM DTPA at 4°C. At this time the thiol concentration of the S-nitrosoalbumin was checked to ensure that only vicinal thiols had been reduced.

A solution of 100mM S-nitrosocysteine (SNOC) was then prepared by addition of 100mM $NaNO_2$ to 100mM L-cysteine. The concentration of the SNOC was established by monitoring the compound at 335nm using an extinction coefficient of $900M^{-1}cm^{-1}$.

A 10mg/mL solution of reduced HSA was then incubated with 10mM SNOC for 30 minutes in the dark (all dilutions made with PBS+DTPA). 10mM N-ethylmaleimide was then incubated with the solution for a further 30 minutes in the dark in order to block all thiol groups. The solution was then dialysed against 3×3L of PBS+DTPA at 4°C in the dark. After 48 hours dialysis, the S-nitrosated albumin was analysed for S-nitrosothiol, HgSNOCS, nitrite and low molecular weight S-nitrosothiol concentration and the remainder aliquotted out and stored at -80°C until use.

4.2.4. Synthesis of mercury-stable NO carrying albumin

A 20mg/mL solution of fatty acid free, >99% pure human serum albumin was made up in PBS. The thiol groups were reduced using 0.5mM dithiothreitol (DTT) in the dark for 2 hours and then dialysed against 3×3L PBS+100μM DTPA at 4°C. At this time the thiol concentration of the albumin was checked to ensure that only vicinal thiols had been reduced. The albumin was then exposed to 10mM N-ethylmaleimide (NEM, final concentration) for 10 minutes at room temperature to block all thiol groups.

A 100mM solution of the NO-donor, DETA-NONOate, was made up in 10mM NaOH. This was added to the NEM-blocked albumin to give a final concentration of 1mM DETA-NONOate and was incubated for 4 hours at 37°C under constant rotation. After 4 hours incubation the albumin was dialysed against 3×3L PBS+100μM DTPA at 4°C to remove the excess NEM and DETA-NONOate. The plasma was then analysed for S-nitrosothiols, mercury-stable NO-carrying species (HgSNOCS), low molecular weight NO-carrying species and nitrite using the techniques described in earlier chapters.

In a separate set of experiments, an identical protocol was followed, except that NEM was not added to the albumin. This control experiment was designed to ensure that any observed mercury-stable nitrosation was not simply an associated of NO with NEM.

4.2.4.1. Synthesis of "mock nitrosated" albumin

"Mock nitrosated" plasma was synthesised by the same method as used to prepare mercury-stable NO-carrying albumin (HgSNOCA) except that, instead of incubation with DETA-NONOate, albumin was exposed only to the vehicle, 10mM NaOH.

4.2.5. Saturation of mercury-stable NO-carrying species formation on albumin

The method was the same as described above for the formation of mercury-stable NO-carrying albumin (HgSNOCA), except that a 1mg/mL solution of human serum albumin was made up. Also, as well as using the 1mM DETA-NONOate final concentration for nitrosation of albumin, a 50μM concentration of DETA-NONOate final concentration was also used for comparison purposes.

Either 50μM or 1mM DETA-NONOate was incubated with albumin for 0, 1, 3.5, 8 or 24hr. At these time points, samples were transferred to dialysis tubing and dialysed against 3×3L PBS+100μM DTPA at 4°C to stop the reaction and remove NEM and DETA-NONOate.

4.2.6. Effect of incubation of S-nitrosoalbumin and mercury-stable NO-carrying plasma on platelet aggregation

HgSNOCA was incubated with human platelet rich plasma and the platelet aggregation induced by 8μM ADP was compared to that induced without incubation. The maximum

concentration of HgSNOCA that could be prepared with 20mg/mL HSA was 4μM, so large volumes of HgSNOCA were needed to incubate with the PRP to establish even nanomolar concentrations of HgSNOCS. Therefore a control experiment was carried out using "mock" nitrosated albumin. This was created by the same method as for synthesis of HgSNOCA as described in section 5.2.4.1. However, instead of incubating the thiol blocked albumin with DETA-NONOate "mock" nitrosated albumin was incubated with the DETA-NONOate vehicle 10mM NaOH. The "mock" nitrosated albumin could therefore distinguish between the effects of the incubation of platelets with a large concentration of albumin and the effect of the NO group carried by HgSNOCA.

Platelets were isolated from a single volunteer on multiple occasions and the platelet count compared on each occasion. The platelet count was found to be 240,000±20,000platelets/μL. PRP was prepared as described in section 2.2.7.

Two concentrations and exposure times of HgSNOCA were used to assay its bioactivity on platelet aggregation: 650nM and 2μM; 3mins and 30mins. For the 2μM HgSNOCA incubation 120μL of PRP was diluted with 120μL of 4μM HgSNOCA or 120μL of "mock" nitrosated albumin. For the 650nM HgSNOCA incubations 120μL PRP was diluted with 39μL of either 4μM HgSNOCA or "mock" nitrosated albumin and 81μL PPP. A control experiment was also carried out in which the 120μL of PRP was diluted with 120μL PPP.

For the 30 minute exposure experiments the PRP was incubated with HgSNOCA/"mock" nitrosated plasma for 20 minutes at room temperature and then transferred to the 37C heating block of the platelet aggregometer for 10 minutes and then assayed. For the 3 minute exposure time the 120μL PRP aliquots were incubated in the platelet aggregometer for 7 minutes with the 81μL of PPP. After 7 minutes the 39μL of HgSNOCA/"mock nitrosated" albumin was added for 3 minutes and the platelet aggregation was then assayed. Platelet aggregation was assayed as described previously.

4.2.7. Displacement of unconjugated bilirubin from mercury-stable NO-carrying plasma and albumin

This method was taken from Tietz[248]. An 11.2% caffeine/sodium benzoate (50:50 cafeine:sodium benzoate) + 0.1% EDTA + 5.6% anhydrous sodium acetate solution was made up in milliQ water. This solution is used to remove unconjugated bilirubin from albumin in many standard biochemical assays for determination of unconjugated/total bilirubin. 50μL of HgSNOCA/plasma was added to 500μL of the caffeine/sodium benzoate solution, vortexed and incubated at room temperature for 30mins. After incubation the plasma/caffeine/benzoate solution was transferred to a 30KDa molecular weight cut off filter and centrifuged at 10 000g for 45mins at room temperature. The filtrate was injected into the nitric oxide analyser and assayed for S-nitrosothiol/HgSNOCS. The pellet was then resuspended in PBS+100μM DTPA and the S-nitrosothiol/HgSNOCS concentration assayed. Aliquots of caffeine/sodium benzoate treated plasma/HgSNOCA were also injected into the NOA without passing through the filter.

4.2.8. Stability of Hg-stable nitrosated plasma to cyanide and thiol exposure

The method for assessment of Hg-stable NO-carrying species stability to cyanide was taken from the method of Yang[122]. Blood was collected into a solution of 1mM NEM/ 100μM DTPA and as described in section 2.3.6. to block the thiol groups and chelate any metal ions. Samples were spun for 1 minute and the plasma removed.

Three 200μL aliquots of plasma were then taken into ependorf tubes and treated as follows:

Tube 1: 200μL plasma + 300μL PBS/NEM/DTPA

Tube 2: 200μL plasma + 50μL $HgCl_2$ + 250μL PBS/NEM/DTPA

Tube 3: 200μL plasma + 50μL $HgCl_2$ + 250μL $K_3Fe(CN)_6$

(The PBS/NEM/DTPA solution constituted 1mM NEM and 100μM DTPA)

Tube 1 was left 30 minutes, tube 2 for 60 minutes and tube 3 for 90 minutes before assay. After the allotted incubation time, the aliquots were added to a Sepahdex G25, PD10 size exclusion column (Amersham Pharma. Biotech, UK) pre-washed with PBS/NEM/DTPA and allowed to completely absorb. Once absorbed, sequential 1mL PBS/NEM/DTPA aliquots were used to elute the column and each 1mL fractionate was collected, treated

with sulphanilamide and injected into a KI/acetic acid solution as described in section 2.2.6. Therefore, the signal generated from:

Tube 1 = S-nitrosothiol + N-nitrosamine + iron-nitrosyl

Tube 2 = N-nitrosamine + iron-nitrosyl

Tube 3 = N-nitrosamine

4.3. Results

4.3.1. Synthesis of mercury-stable NO-carrying species in human plasma

Exposure of NEM pre-treated human plasma to 1mM DETA-NONOate for 4 hours at 37°C resulted in the formation of only trace amounts of HgSNOCS (below detection limit) and was referred to as nitrosated mercury-stable nitrosated plasma (HgSNP). There was no trace of any concomitant S-nitrosothiol formation. After reaction with NEM, the thiol concentration was always checked to ensure that all thiol residues had been blocked.

4.3.2. Determination of HgSNOCS molecular weight

4.3.2.1. Molecular weight cut off filters

30KDa and 100KDa molecular weight cut off filters were used to give a rough estimation of the size of HgSNOCS in mercury-stable nitrosated plasma (HgSNP). The pellet retained in the filter was re-suspended in PBS/NEM/DTPA and the HgSNOCS concentration was measured and compared to that in the eluate from each column. The HgSNOCS concentration recorded in both the eluate and re-suspended pellet was expressed as the percentage of the concentration recorded from an equal volume of HgSNP analysed directly for HgSNOCS concentration without passing through a molecular weight cut off filter. It was found that ~95% of the HgSNOCS in HgSNP were

found in the pellet of both the 30KDa and 100KDa whilst only the remaining ~5% passed through either filter (Figure 32). This data therefore suggests that the HgSNOCS in HgSNP is larger than 100KDa. However, molecular weight cut off filters separate molecules on the basis of shape as well as size and the experiment was not carried out under reducing conditions so the presence of dimerised proteins was possible. Therefore proteins approaching 100KDa were not discounted at this stage.

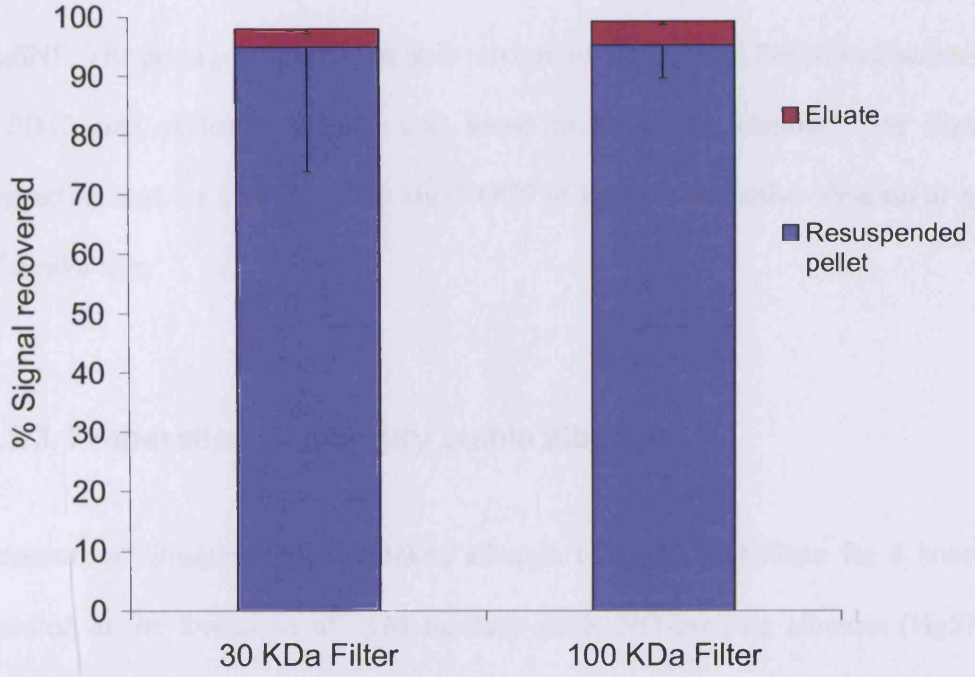


Figure 32. Quantity of HgSNOCS recovered in the eluate and pellet when HgSNP is passed through a 30KDa and 100KDa molecular weight cut off filter. Only trace HgNOCS were found in the eluate from either the 30KDa or 100KDa filters indicating that the HgSNOCS has a molecular weight greater than 100KDa. (n=3)

4.3.2.2. Fractionation profile of compared to S-nitrosoalbumin

Given that S-nitrosoalbumin detection is routinely carried out in the laboratory and albumin has a known molecular weight of ~68 000 the relative fractionation profile of S-nitrosoalbumin from a size exclusion column was compared to that of HgSNOCS in HgSNP. The passage of a stoiciometric mixture of HgSNP and S-nitrosoalbumin through a PD10 size exclusion column was found to be almost identical (see Figure 33), suggesting that the identity of the HgSNOCS in HgSNP was either albumin or a protein of similar size.

4.3.3. Preparation of mercury stable albumin

Exposure of 20mg/mL NEM-blocked albumin to DETA-NONOate for 4 hours, 37°C resulted in the formation of 2μM mercury-stable NO-carrying albumin (HgSNOCA). Albumin exposed to non-NEM-blocked albumin resulted in formation of 83% HgSNOCA and 17% S-nitrosoalbumin demonstrating that HgSNA formation was independent of NEM nitrosation.

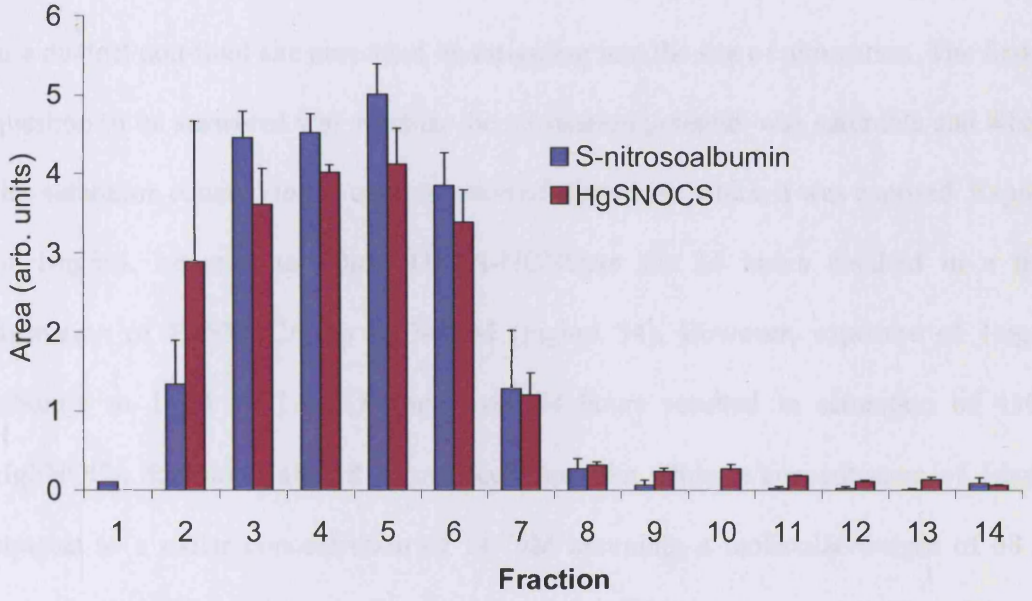


Figure 33. Fractionation profile of S-nitrosoalbumin and HgSNOCS from HgSNP upon co-elution from a PD10 sepharose size exclusion column compared to that of S-nitrosoalbumin. S-nitrosoalbumin had an almost identical elution profile to the HgSNOCS. (n=3)

4.3.4. Saturation of albumin mercury-stable nitrosation potential

The discovery that albumin can be nitrosated not only by S-nitrosation on cys-34 but also at a distinct non-thiol site prompted investigation into the site of nitrosation. The first key question to be answered was whether the nitrosation potential was saturable and whether this saturation equated to the concentration of albumin to which it was exposed. Exposure of 1mg/mL albumin to 50μM DETA-NONOate for 24 hours resulted in a linear formation of HgSNOCA up to 340nM (Figure 34). However, exposure of 1mg/mL albumin to 1mM DETA-NONOate over 24 hours resulted in saturation of 450nM HgSNOCA formation after 8 hours incubation. An albumin concentration of 1mg/mL equates to a molar concentration of 14.7μM assuming a molecular weight of 68 000 Daltons. As the saturation of formation of HgSNOCA equates to only 3% of the total albumin concentration this suggests that either 3% of albumin protein in our assay exist in a form susceptible to nitrosation or 3% of albumin proteins are bound to the nitrosated species. Given that albumin is such an important plasma transport molecule it is hypothesised that although the albumin source used was de-fatted and 99% pure, tightly bound molecules are likely to remain attached to albumin. Possible tightly bound albumin associated factors were therefore considered for investigation. Bilirubin has been shown to be susceptible to N-nitrosation and to decompose S-nitrosothiols[249].

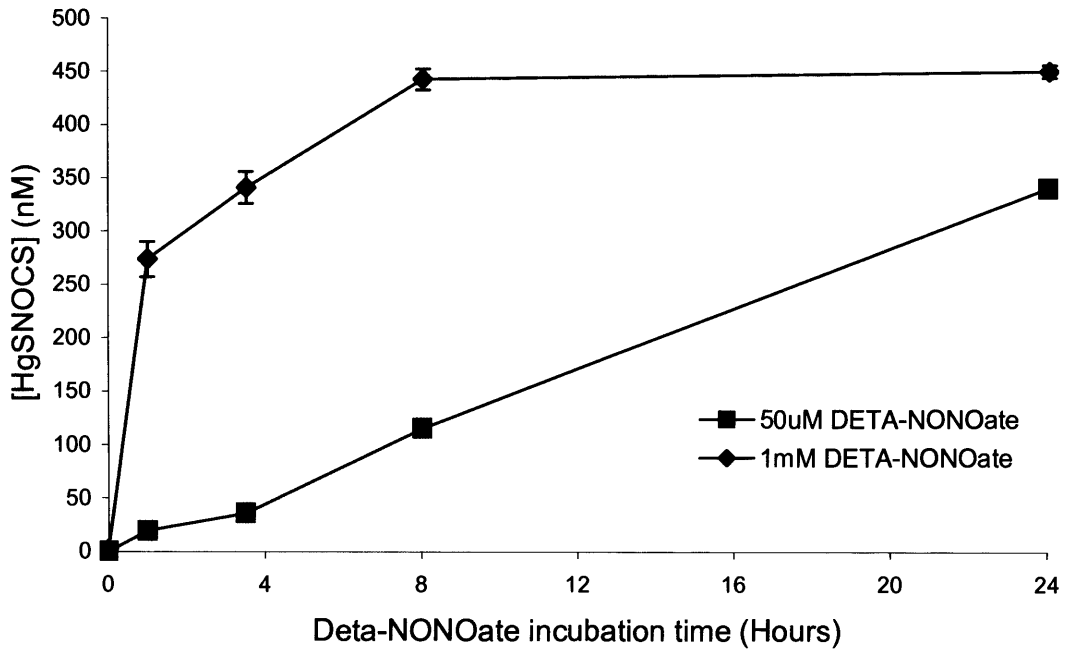


Figure 34. Saturation of the formation of HgSNOCS in 1mg/mL solution of albumin pre-treated with NEM to block thiol groups. (n=3)

4.3.5. Stability of the mercury-stable nitrosated species bond

4.3.5.1. Stability at room temperature

Mercury-stable nitrosated species were stable at room temperature for 48 hours. This was validated in 3 separate experiments in which a sample of the synthesised nitrosated albumin (HgSNOCA) was left on the bench at room temperature. The concentration of HgSNOCS was then assessed by chemiluminescent analysis immediately after synthesis, and also after 24 and 48 hours. There was no degredation in the signal recorded after either 24 or 48 hours (see figure 35).

4.3.5.2. Stability to cyanide and thiol exposure

Iron-nitrosyl bonds are susceptible to cyanide displacement and this reaction can be easily used to destroy iron-nitrosyl bonds. However, cyanide is also known to cause a chemiluminescent signal on reaction with ozone and therefore excess cyanide (un-reacted with iron) must be removed before analysis in the NO analyser. Cyanide can be separated from proteins using a PD10 size exclusion column on the basis of the large molecular weight difference between protein and cyanide. HgSNP was therefore added to a PD10 column with or without prior incubation with $K_3Fe(CN)_6$ and fractions eluted were collected and analysed for HgSNOCS concentration. No difference was found in the

elution profile of HgSNOCS from the PD10 column with or without cyanide pretreatment indicating that the HgSNOCS in HgSNP is not an iron-nitrosyl (see Figure 36).

HgSNP was also treated with 1mM GSH over a 4 hour timeline and no degradation of signal was recorded over this time period.

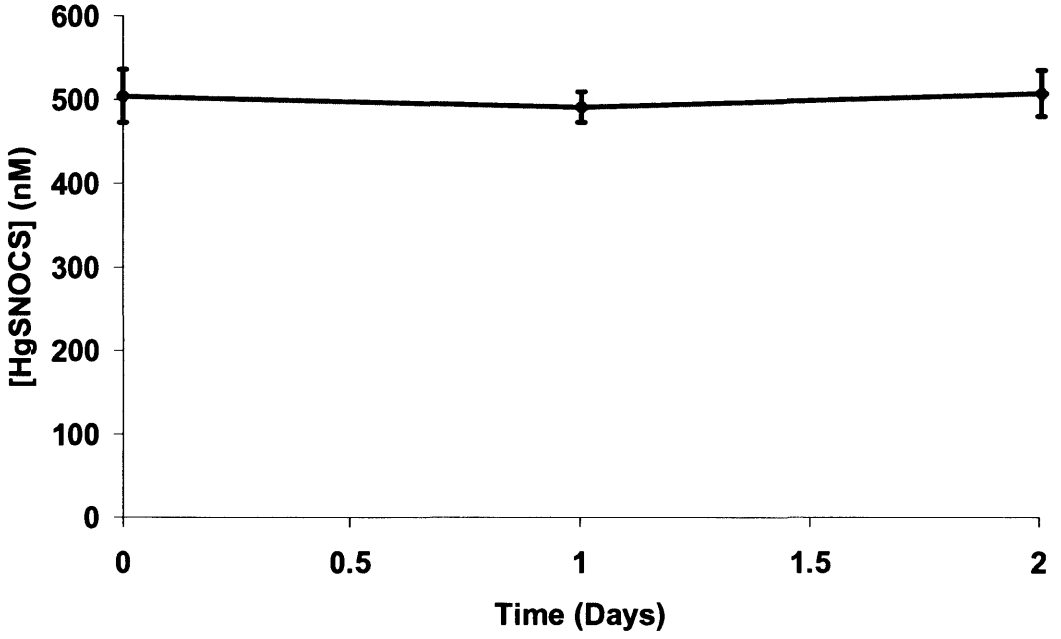


Figure 35. Stability of synthesised mercury-stable plasma at room temperature over a 48 hour time period. There was no degredation of [HgSNOCS] recorded over a 48 hour time period. P=0.89. Statistics performed using one-way ANOVA.

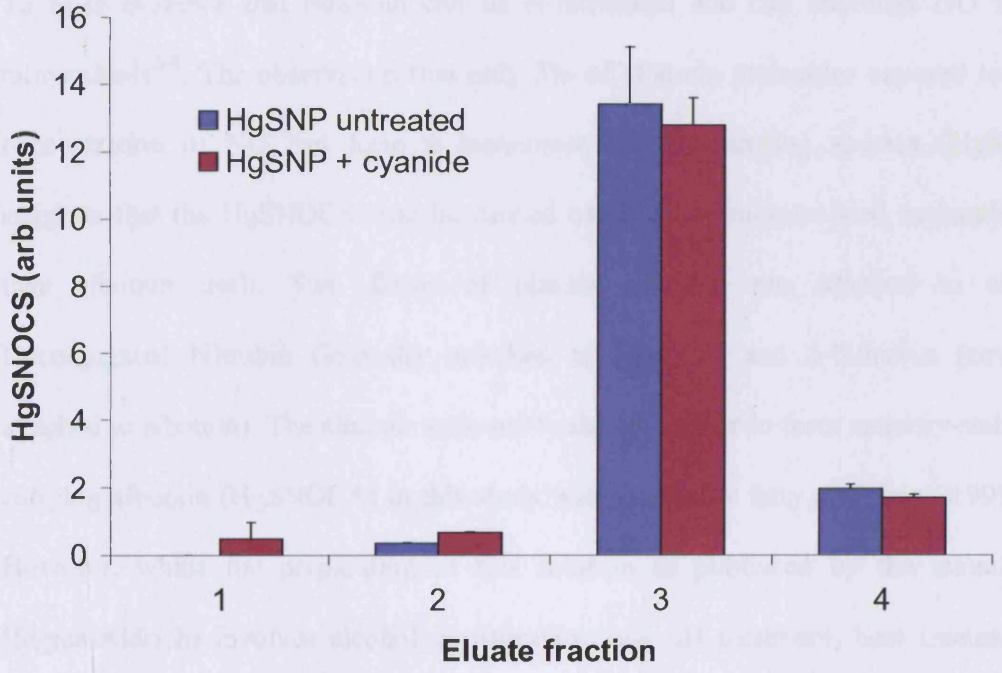


Figure 36. Stability of HgSNOCS in the presence of cyanide. Concentration of mercury-stable NO-carrying species (HgSNOCS) measured in serial fractions of mercury-stable nitrosated plasma (HgSNP) eluted from a PD10 size exclusion column in the presence and absence of cyanide. Exposure to cyanide had no effect on the stability of HgSNP indicating that the nature of the X-NO bond is not an iron-nitrosyl species. (n=3)

4.3.6. Bilirubin and N-nitrosation

There is evidence that bilirubin can be N-nitrosated and can scavenge NO from S-nitrosothiols[249]. The observation that only 3% of albumin molecules exposed to a high concentration of NO can form a mercury-stable NO-carrying species (HgSNOCS) suggests that the HgSNOCS may be carried on an albumin-associated molecule rather than albumin itself. Two forms of plasma bilirubin are attached to albumin: Unconjugated bilirubin (ionically attached to albumin) and δ-bilirubin (covalently attached to albumin). The albumin exposed to the NO-donor to form mercury-stable NO-carrying albumin (HgSNOCA) in this study was essentially fatty acid-free, ≥99% pure. However, whilst the preparation of this solution as published by the manufacturer (Sigma-Aldrich) involves alcohol denaturation, low pH treatment, heat treatment and ammonium sulphate exposure, any of which could remove unconjugated bilirubin. However, none of these purification steps would be expected to remove δ-bilirubin.

Caffeine-sodium benzoate solution is used to displace unconjugated bilirubin from its albumin-binding site for assessment of direct and indirect bilirubin concentration in routine chemical pathology assays. However, when injected into the NO analyser it was found that the caffeine/sodium benzoate solution produced a chemiluminescent signal.

4.3.7. Effect of mercury-stable NO-carrying albumin on platelet aggregation

Given the concentration of HgSNOCS in the plasma of endotoxaemic cirrhotic rats, and the potential reservoir of NO this represents, the effect of mercury-stable nitrosated plasma (HgSNP) on platelet aggregation was examined. Plasma itself may have an effect on platelet aggregation, so a control experiment was also carried out in which the effect of "mock-nitrosated" plasma on platelet aggregation was assessed. "Mock-nitrosated" plasma was exposed to the DETA-NONOate vehicle, 10mM NaOH instead of DETA-NONOate and then otherwise dialysed and treated as the HgSNOCA. No S-nitrosothiol or HgSNOCS were detected in "mock nitrosated" plasma. The effect of HgSNP on platelet aggregation was also compared to that of S-nitrosoalbumin

It was found that 650nM S-nitrosoalbumin (the concentration observed in endotoxaemic cirrhotic rats) inhibited platelet aggregation when incubated with platelets for 30 minutes (195.4±17arb. vs. 88.51±42arb. in PBS incubated and S-nitrosoablbumin treated platelets respectively, Figure 37). 3 minute incubation of platelets with 650nM S-nitrosoablumin had no statistically significant effect on platelet aggregation.

Incubation of "mock nitrosated" plasma had no effect on platelet aggregation following 3 or 30 minute incubation with platelets. However, incubation of 650nM HgSNP resulted in an identical inhibition of platelet aggregation as observed following incubation with an equal concentration of S-nitrosoalbumin. Incubation of platelets with 650nM HgSNP

resulted in a decrease in observed aggregation compared to mock nitrosated albumin: 103.7±39.5arb. vs. 193.2±16arb respectively.

The stability of HgSNOCA in solution makes the observed anti-platelet activity hard to understand. There may be an enzymic mechanism which decomposes the NO from the albumin, but there was insufficient time to analyse the effect of platelet incubation on HgSNOCA stability. The time-dependence of HgSNOCA incubation on anti-platelet activity indicates that decomposition of the albumin-NO bond is a relatively slow reaction.

The activity of HgSNP deserves further investigation, especially considering that the 2μM concentration of HgSNP shown to inhibit platelet aggregation corresponds to the pathological concentration observed in endotoxaemic cirrhotic rats, which themselves show dysfunctional platelet aggregation.

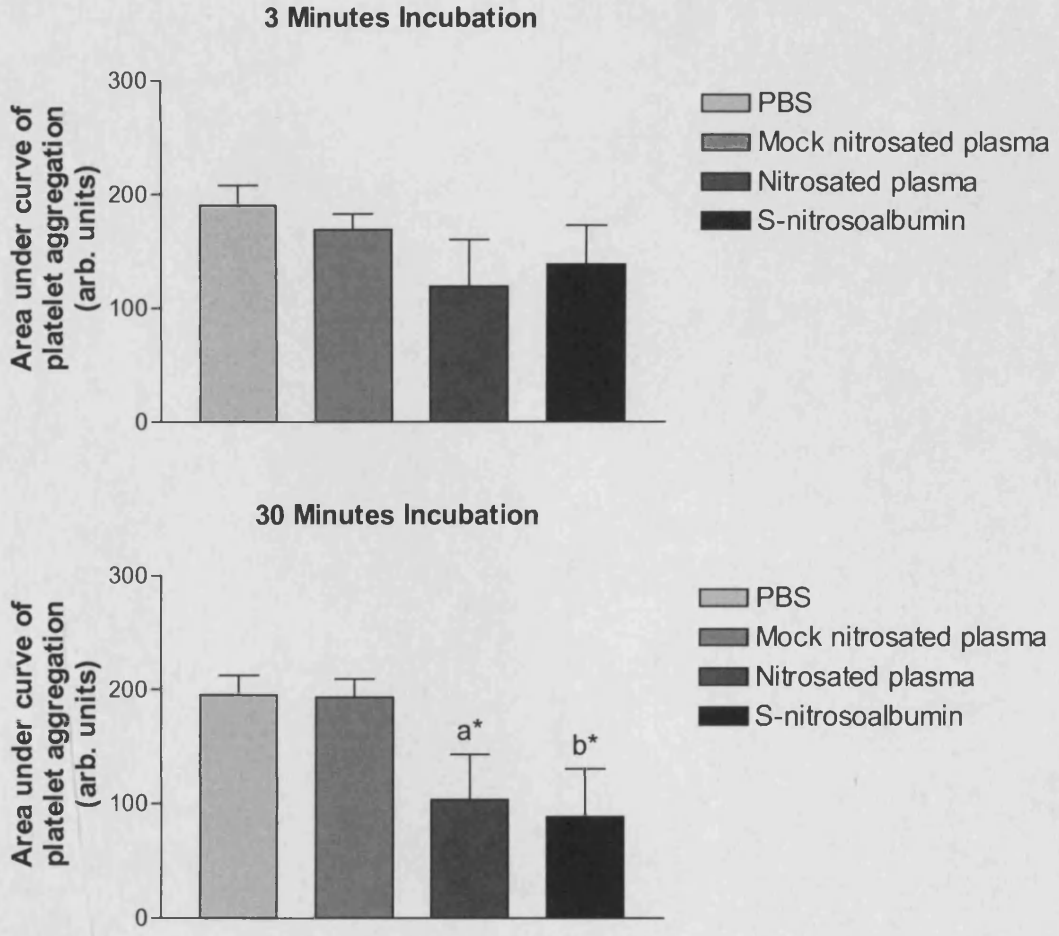


Figure 37. Comparison of the effect of S-nitrosoalbumin and mercury-stable-nitrosated plasma (HgSNP) on platelet aggregation in human platelet rich plasma (PRP). Incubation of PRP with 650nM HgSNP (30mins) resulted in a significant inhibition of platelet aggregation vs. mock nitrosated plasma after 30 minute incubation ($p<0.05$). This inhibition of platelet aggregation was identical to that from an equivalent concentration of S-nitrosoalbumin. $n \geq 4$ for all conditions studied. Statistics carried out by repeated measduresb one-way ANOVA ($P=0.051$ and $P=0.181$ for 3 and 30 minutes respectively) with Newman-Keuls post-hoc comparison. a*=$p<0.05$ vs. mock nitrosated plasma, b*=$p<0.05$ vs. PBS.

4.4. Discussion

Whilst the identity of the HgSNOCS has not been fully elucidated, some major properties have been determined. The molecular weight indicated by the passage of the HgSNOCS through a size exclusion column is almost identical to that of albumin. Furthermore, exposure of albumin to an NO donor results in a similar formation of HgSNOCS (~2μM HgSNOCS after 20mg/mL HSA exposure to 1mM DETA-NONOate, 37°C, 4hr) as found when plasma is exposed to NO (~4μM HgSNOCS after plasma {40mg/mL HSA} exposure to 1mM DETA-NONOate, 37°C, 4hr). However, the saturation of HgSNOCS formation on albumin is only equal to ~3% of albumin molecules available despite exposure to a vast molar excess of NO. This indicates that the formation of HgSNOCS is relatively specific and not a random effect of exposure of large quantities of NO to albumin *in vitro*. The theory that this saturation of mercury-stable nitrosation is due to N-nitrosation of albumin-bound bilirubin has been tested briefly and no evidence for this could be found. However, this theory deserves further analysis. It is also possible that another albumin-bound molecule could be nitrosated or that only 3% of albumin molecules exist in a form in which they can be nitrosated. As it has been shown that the HgSNOCS formed is not S-nitrosothiol or iron nitrosyl, and given it's innate stability in plasma, the most likely explanation is that it is a secondary nitrosamine, as also deduced by Feelisch et.al. in normal plasma[133]. A further experiment, would be illuminating to carry out, would be to examine the stability of the HgSNOCS synthesised in a range of biological fluids. An experiment in which HgSNOCS were incubated with whole blood,

NEM-treated plasma and native plasma over time could determine if there is a mechanism of active release of NO from HgSNOCS and localise a site for this.

Surprisingly, it has also been found that mercury-stable nitrosated plasma (HgSNP) has NO-like anti-platelet activity at pathologically relevant concentration. Approximately 2μM HgSNOCS are found in the plasma of endotoxaemic cirrhotic rats; platelets isolated from these individuals are extremely hyporesponsive to ADP-induced platelet aggregation. 650nM HgSNP also has a significant inhibitory effect on platelet aggregation and this activity is dependent on the previous exposure of the plasma to NO. This observation implies that there is a second reservoir of NO stabilised bioactivity in the circulation other than S-nitrosothiol. Furthermore, this reservoir is found in greater concentration than S-nitrosothiol in the circulation. Whilst it is expected that NO is released or donated by HgSNP and HgSNOCA in this discussion, the lack of any effect of ODQ makes HgSNOCS unique in terms of NO donors. No other NO donor species is completely independent of cGMP. It is therefore still possible that the observed effects are entirely independent of NO and experiments could be carried out to assess this by savenging NO with haemoglobin or directly measuring NO release from HgSNOCS with a fluorescence marker.

In conclusion, the discovery that signal previously attributed to S-nitrosothiol formation in the chemiluminescent detection of S-nitrosothiol species in the plasma is non-mercury decomposable, and therefore not S-nitrosothiol, has led to speculation as to the nature of this species. Very high concentrations of these mercury-stable NO-carrying species

(HgSNOCS) are found in endotoxaemic cirrhotic rats and these rats also show profoundly dysfunctional platelet aggregation. The HgSNOCS in plasma is high molecular weight and has been shown in this study to be associated to albumin or a closely related protein. Further, artificially generated HgSNOCS have a NO-like inhibitory effect on platelet aggregation, similar to that elicited by S-nitrosothiols, the NO-dependence of which is yet to be proven. However, HgSNOCS are present in the plasma at higher concentration than S-nitrosothiols and, unlike S-nitrosothiols, a pathologically relevant concentration of HgSNOCS can influence platelet aggregation. It is therefore speculated that HgSNOCS may be as important a reservoir of NO-like activity as S-nitrosothiols in endotoxaemia and cirrhosis in rats.

Chapter 5: Effect of low molecular weight antioxidants on vascular function in the cirrhotic rat

5.1. Introduction

As part of our programme to investigate the therapeutic potential of low molecular weight thiols in cirrhosis I also characterised the effect of lipoic acid on vascular function. Previous results from our group have demonstrated an improvement of haemodynamic parameters and kidney function in rats with hyperdynamic circulation and/or hepatorenal syndrome when low molecular weight antioxidants were administered prior to or shortly after the induction of liver disease[223,224,229]. One of the key steps in the development of the hyperdynamic circulation is the development of vascular hyporesponsiveness to vasoconstrictors. Vascular dysfunction is observed in isolated aortic rings from bile duct ligated (BDL) cirrhotic rats showing markedly lower contraction in response to phenylepherine stimulation compared to sham controls[206,207,208]. Since LA has been shown to prevent the development of vasodilation in the context of the hyperdynamic circulation[229], I hypothesised that this effect would be on the vasculature. Thus, the effect of thiol antioxidants on vascular dysfunction in cirrhotic rats was examined in isolated aortic rings.

The responsiveness of aortic rings from normal and BDL cirrhotic rats to acetylcholine-induced vasodilatation was also assessed, as there is some discrepancy in the literature as to whether cirrhotic rat vascular tissue is hypo-responsive to acetylcholine-induced

relaxation or the same as that from sham control rats. Renal dysfunction was assessed by creatinine clearance over 24 hours. When this project commenced it was generally believed that S-nitrosothiols are arterial vasodilators. We now know from the work of Orie *et.al.*[250] that S-nitrosoalbumin is predominantly a venodilator and thus this initial hypothesis is unlikely to be true.

The potential mechanism of thiol antioxidants on the hyperdynamic circulation initially proposed at the start of this project was that administration of lipoic acid would accelerate the decomposition of S-nitrosothiols and prevent vasodilation or normalise vascular function.

5.2. Materials and Methods

5.2.1. Animals

See chapter 2

5.2.1.1. Induction of biliary cirrhosis - Bile Duct Ligation (BDL)

See chapter 2

5.2.1.2. Administration of lipoic acid

Lipoic acid was administered in the drinking water at a concentration of 0.1% to which the rats were given free access for 7 days (days 18-24 post-bile duct ligation). Lipoic acid was prepared by first dissolving in a minimum volume of 1M NaOH and then made up to volume in distilled water. The pH was then titrated back to pH 7.0 by addition of 1M HCl.

5.2.1.3. Metabolic cages

For 24 hour urine collections, rats were placed in metabolic cages at least 24 hours before the start of the collection period. Animals were given free access to powdered RM1 chow and water/0.1% lipoic acid drinking water over this time.

5.2.1.4. Collection of blood

5.2.1.4.1. For measurement of creatinine, urea and LFTs

Blood was collected from the same animals from which plasma was taken for measurement of nitrosothiol/ iron-nitrosyl concentration. A fresh 5mL syringe was placed on the 23G needle after heparinised blood had been taken and blood was drawn until complete exsanguination. This was dispensed into a 10mL EDTA vacutainer and the blood stored at 4°C for 10mins. Blood was then centrifuged at 3000rpm, 10mins, 4°C. After centrifugation the plasma was removed and stored in 0.5mL aliquots at -80°C until analysis.

5.2.1.4.2. For measurement of S-nitrosothiols

Animals were anaesthetised with pentobarbital and exsanguinated through the abdominal aorta using a 23G butterfly needle. Blood was collected into a 5mL syringe containing 20U heparin and two 900μL aliquots added to two eppendorf tubes containing 50μL 200mM N-ethyl-maleimide (0.9% NaCl) and 50μL 2mM DTPA (0.9% NaCl) and inverted to mix. The tubes were then spun at *13 000 rpm* directly for 1min and the plasma removed for immediate analysis.

5.2.2. Biochemical analysis

5.2.2.1. Glomerular filtration rate (GFR) - Creatinine clearance

Serum and urine creatinine was measured by a kinetic colorimetric method (Hitachi Auto-analyser, Japan). This method involves converting bilirubin to biliverdin with potassium hexacyanoferrate (III), and uses a dual wavelength absorption method, eliminating interference from bilirubin.

$$\textit{Creatinine clearance (ml/min)} = \frac{\textit{urine volume (ml) x urine creatinine (}\mu\textit{M)}}{\textit{plasma creatinine (}\mu\textit{M) x time (mins)}}$$

5.2.3. Response of isolated aortic rings to vasoactive pharmacological agents

5.2.3.1. Tissue preparation and mounting rings in organ bath

Animals were anaesthetised with pentobarbital and exsanguinated from the abdominal aorta. The descending thoracic aorta was excised from the diaphragm to the aortic arch and placed immediately in oxygenated Krebs buffer (NaCl: 112mM, KCl: 5mM, $CaCl_2$: 1.8mM, $MgCl_2$: 1mM, $NaHCO_3$: 25mM, KH_2PO_4: 0.5mM, NaH_2PO_4: 0.5mM, Glucose: 10mM). Excess connective tissue was removed but adventitia was retained before cutting

into 4-5mm length rings. Vessels were then mounted in an ADInstruments 4-chamber organ bath system (ADI, Hastings, UK) by suspending rings from an isometric force transducer by a hook suspended with thread (see Figure 38).

Chambers (25mL) were filled with Krebs buffer (as above) and constantly oxygenated with 95% O_2, 5% CO_2 at 37°C. After excision from the thoracic cavity the tissue was at all times kept in constantly oxygenated Krebs buffer at room temperature until mounting with an average time of 10 mins between excision and mounting. After mounting the rings, the signal of the weight of the ring, hook and thread was zeroed to 0mg tension from the input from the bridge amplifier using ADInstruments Chart® data acquisition software. The rings were then attached to a fixed hook at the bottom of the chamber so it became held loosely between the hook attached to the transducer from above and the fixed hook from below. The Chart® software was then turned on to record, and to each ring was applied 1g of tension.

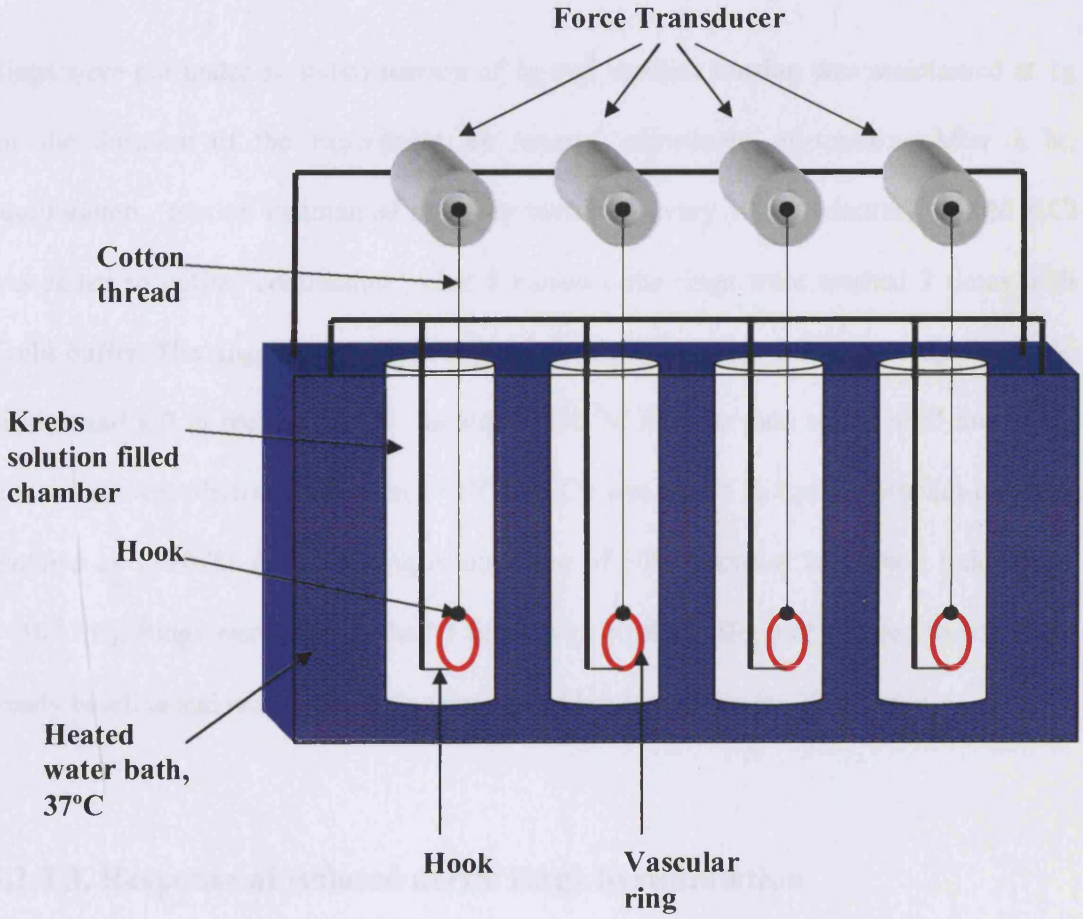


Figure 38. Four chamber organ bath apparatus used for vascular ring studies.

5.2.3.2. Pharmacological pre-conditioning of aortic rings

Rings were put under an initial tension of 1g and baseline tension was maintained at 1g for the duration of the experiment by manual adjustment of tension. After 1 hr. equilibration, (tension maintained at 1g by tweaking every 10-15 minutes), 48mM KCl was added to initiate contraction. After 5 minutes, the rings were washed 3 times with Krebs buffer. The rings were allowed to relax to steady baseline and then readjusted to 1g tension and left to recover for 30 minutes. 1×10^{-7}M PE was then added until maximum contraction was observed and then 1×10^{-6}M ACh was added to test endothelial integrity (defined as 1×10^{-5}M ACh eliciting a minimum of 50% decrease in tension induced by 1×10^{-6} PE). Rings were then washed 3 times with Krebs buffer and allowed to relax to a steady baseline and readjusted to 1g tension and left to recover for 30 minutes.

5.2.3.3. Response of isolated aortic rings to stimulation

After 30 minutes, a concentration response curve to 1×10^{-9}M to 1×10^{-3}M PE was carried out and rings were washed with 3 volumes of Krebs buffer after the final dose of PE. Rings were allowed to settle to baseline and then readjusted to 1g tension. After 60 mins, (tension maintained at 1g by tweaking every 10-15 minutes), 1×10^{-7} – 1×10^{-6} M PE (appropriate concentration to invoke 75-80% of the maximum tension observed in the PE

concentration curve) was added to the well and once 75-80% maximum tension was achieved a concentration response to $-\log_{10}$ 9.0M to $-\log_{10}$ 3.0M ACh was carried out.

5.3. Results

5.3.1. Kidney function

The plasma creatinine concentration was higher in BDL cirrhotic rats than in normal rats (47±1μM normal rats vs. 71±2μM BDL cirrhotic rats), see Figure 39. These values compare closely to previous studies from our laboratory[229]. The creatinine clearance in BDL cirrhotic rats was also slightly lower than in sham control rats as expected in cirrhosis but this difference was not statistically significant (Figure 40). Whilst 7-day lipoic acid supplementation in the water of sham operated rats led to no change in creatinine clearance compared to untreated rats, supplementation to cirrhotic rats (days 18-24) led to a sharp fall in the creatinine clearance compared to untreated cirrhotic controls (Figure 40). Lipoic acid (LA) supplementation in the drinking water of cirrhotic rats therefore appears to impair renal function, as measured by creatinine clearance when administered in the drinking water.

One possible explanation for this observation is that the animals may drink less LA containing water compared to normal drinking water because of its pungent taste leading to dehydration. To test this I compared the water intake of the rats given LA drinking water and compared this to the intake of rats given normal drinking water.

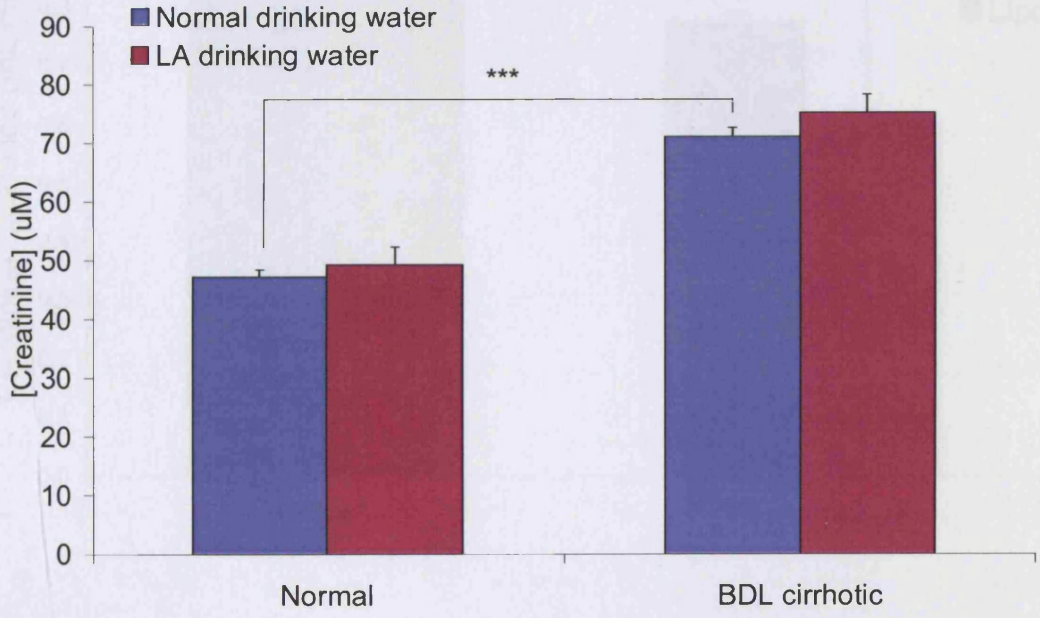


Figure 39. Plasma creatinine concentration in normal and BDL cirrhotic rats given normal drinking water or lipoic acid supplemented drinking water. Those rats given lipoic acid (LA) supplemented drinking water had elevated plasma creatinine concentration indicative of impaired renal function. Statistics carried out by two-way ANOVA ($P<0.0001$) with Newman-Keuls post-hoc comparisons. *** $p<0.001$. n=12 normal sham, n=4 sham+LA, n=5 BDL and n=7 BDL+LA.

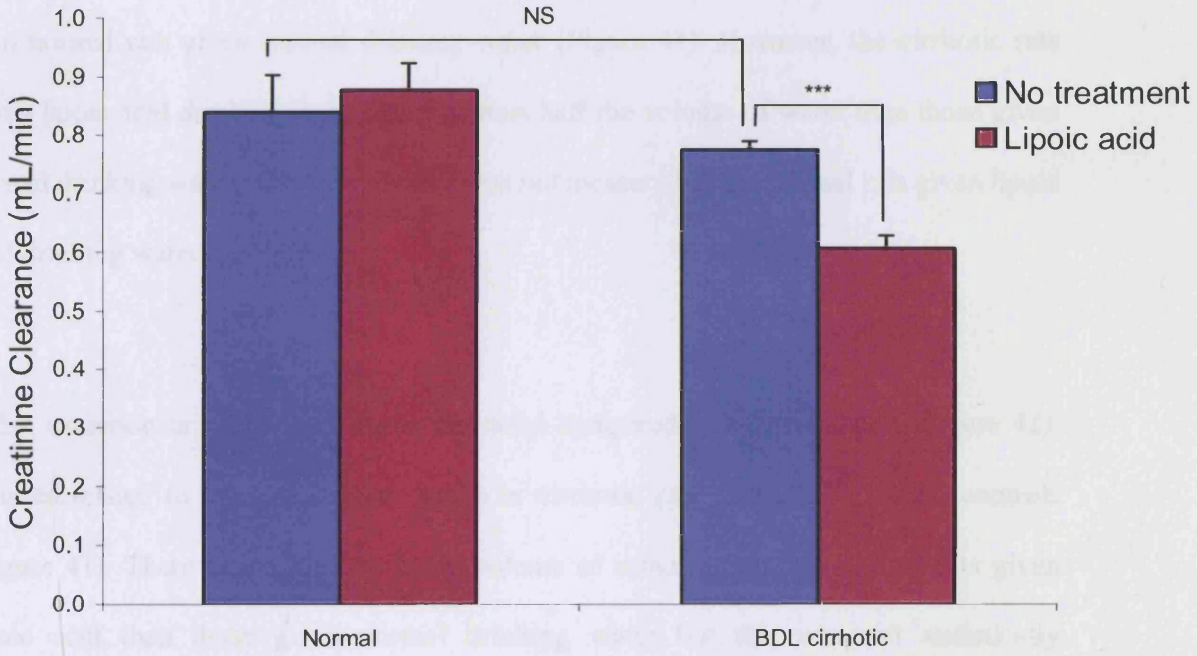


Figure 40. Creatinine clearance in normal and cirrhotic rats with or without lipoic acid supplementation in drinking water. Creatinine clearance was lower, but did not reach significance, in BDL cirrhotic rats given normal drinking water compared to normal rats, contrary to previous reports showing impaired GFR in BDL cirrhotic rats. Lipoic acid (LA) supplementation to the water of normal rats had no effect on creatinine clearance but led to a fall in creatinine clearance when given to cirrhotic rats. Statistics carried out by two-way ANOVA ($P<0.0001$) with Newman-Keuls post-hoc comparisons. NS = not significant, *** = $p<0.001$. n=12 normal sham, n=4 sham+LA, n=5 BDL and n=7 BDL+LA.

5.3.1.1. Water intake and urine excretion

Cirrhotic rats given normal drinking water drank a significantly greater volume of liquid than normal rats given normal drinking water (Figure 41). However, the cirrhotic rats given lipoic acid drinking water drank almost half the volume of water than those given normal drinking water. The water intake was not measured in the normal rats given lipoic acid drinking water.

Urine excretion in cirrhotic rats was increased compared to sham controls (Figure 42). This correlated to increased water intake in cirrhotic rats compared to sham controls (Figure 41). There was a slightly lower volume of urine excreted in normal rats given lipoic acid than those given normal drinking water but this was not statistically significant. Cirrhotic rats given lipoic acid excreted less than half the volume of urine as those given normal drinking water. The relative liquid intake and excretion was consistent in rats ± cirrhosis ± lipoic acid drinking water.

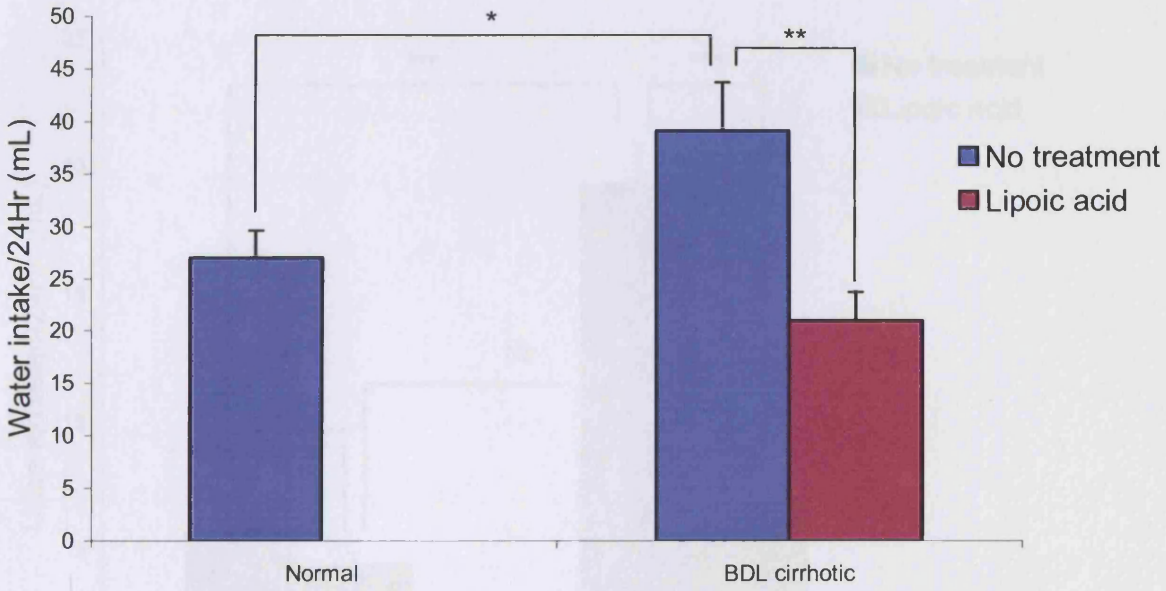


Figure 41. Volume of water consumed over 24 hour period in normal and BDL cirrhotic rats with normal or lipoic acid supplemented drinking water (no data recorded for lipoic acid supplemented sham operated rats). BDL cirrhotic rats given normal drinking water consumed higher liquid volume over the 24 hour measurement period (day 24-25 post bile duct ligation) compared to non-cirrhotic rats. However, cirrhotic rats given lipoic acid supplemented drinking water drank significantly less liquid than when they were given normal drinking water. This change in liquid intake may explain the decreased creatinine clearance observed in BDL cirrhotic rats given lipoic acid supplemented drinking water. Statistics carried out by two-way ANOVA ($P<0.0001$) with Newman-Keuls post-hoc comparisons. * $p<0.05$, ** $p<0.01$. $n=6$ for all groups.

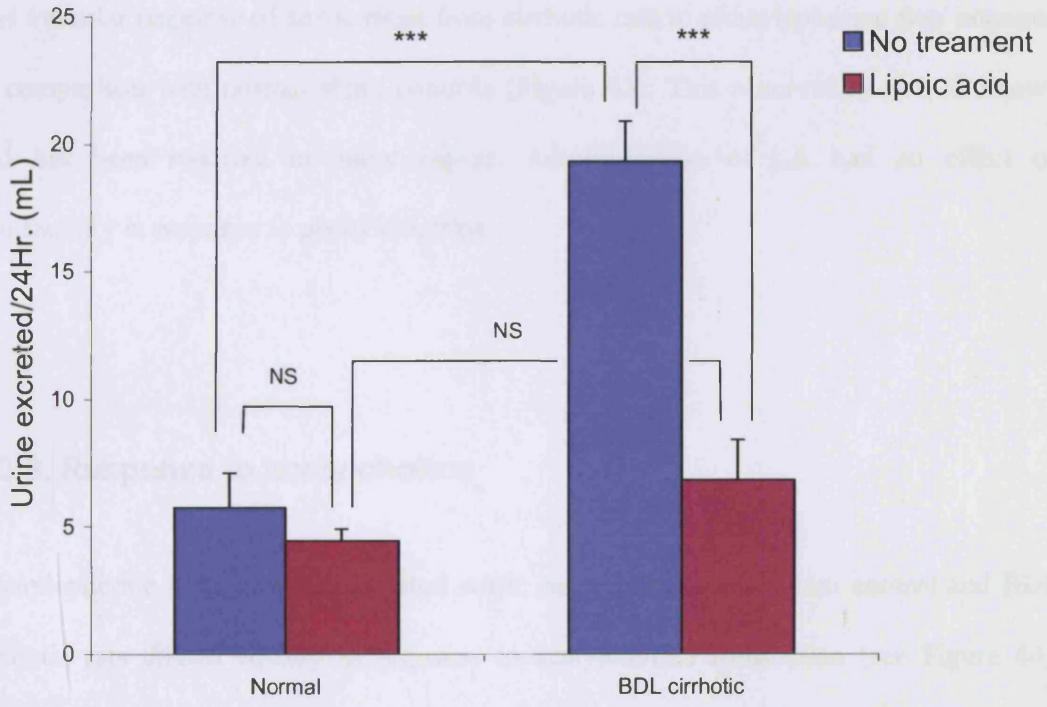


Figure 42. Urine volume excreted over the 24 hours used to measure creatinine clearance in normal and BDL cirrhotic rats given normal or lipoic acid drinking water. Urine excretion was lower in lipoic acid supplemented BDL rats than in rats given normal drinking water consistent with lower water intake (see Figure 41). Lipoic acid supplementation to normal rats resulted in a slightly reduced but non-significant reduction in liquid intake. Statistics carried out by two-way ANOVA ($P<0.0001$) with Newman-Keuls post-hoc comparisons. NS = not significant, *** $p<0.001$. n=6 for all groups.

5.3.2. Response to phenylepherine

The vascular response of aortic rings from cirrhotic rats to phenylepherine was impaired in comparison with normal sham controls (Figure 43). This observation is well known and has been reported in many papers. Administration of LA had no effect on contractility in response to phenylepherine.

5.3.3. Response to acetylcholine

Phenylepherine pre-contracted isolated aortic rings from normal sham control and BDL cirrhotic rats dilated equally in response to acetylcholine stimulation (see Figure 44). Administration of lipoic acid to the drinking water of cirrhotic rats had no effect on acetylcholine induced vasodilatation of isolated aortic rings. These results confirm the findings of other studies in which no difference in isolated tissue response to acetylcholine induced vasodilatation has been observed.

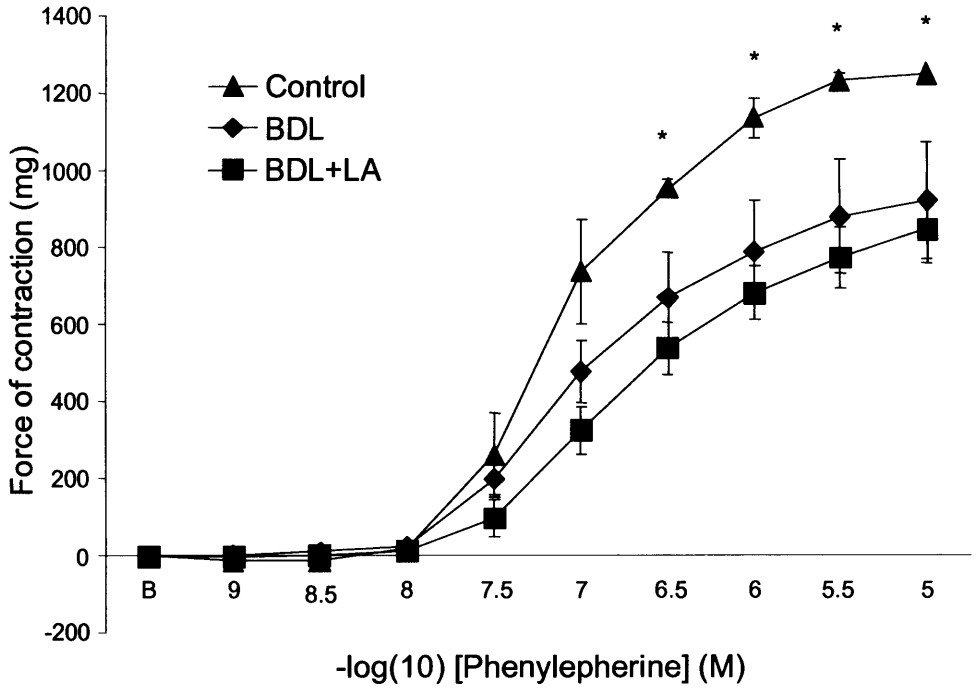


Figure 43. Response of isolated aortic rings from normal and bile duct ligated rats ± lipoic acid drinking water upon phenylepherine stimulation. Isolated aortic rings from BDL cirrhotic rats had lower response to phenylepherine stimulation than those isolated from normal rats. This is consistent with previous experiments and with decreased response to vasoconstrictors observed in cirrhosis. Supplementation of lipoic acid in the drinking water of cirrhotic rats did not improve the response of isolated aortic rings to phenylepherine. Statistics carried out by two-way ANOVA ($P<0.001$) with Newman-Keuls post-hoc comparisons. * = $p<0.05$ control vs. BDL cirrhotic. Normal sham n=3, cirrhotic rats n=6, cirrhotic rats + lipoic acid n=4.

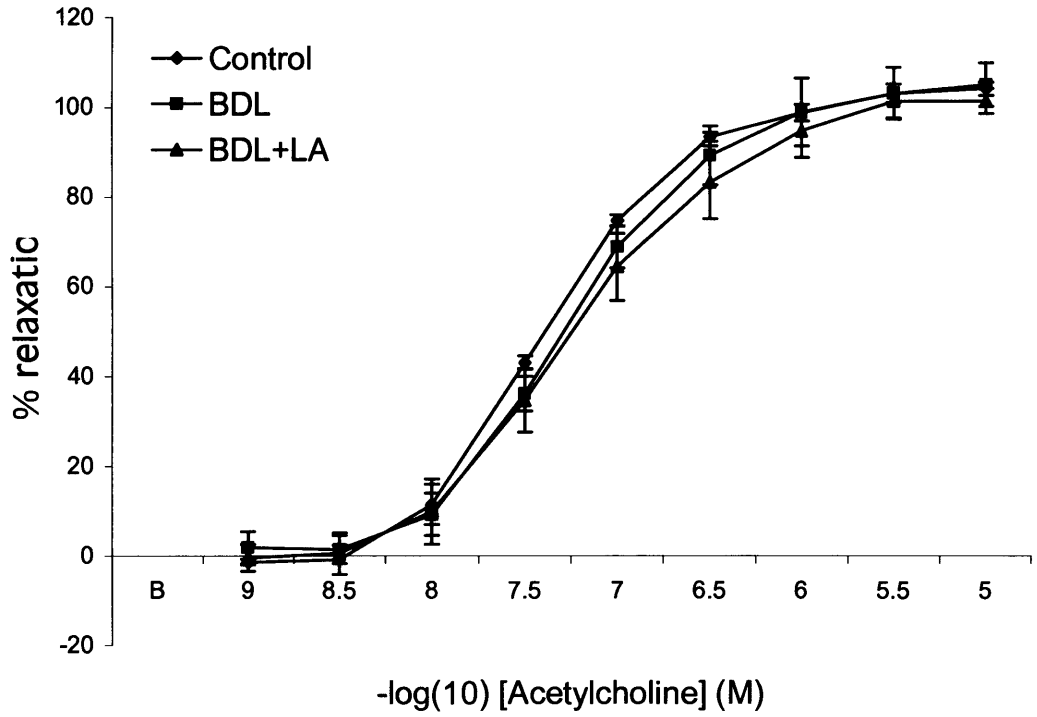


Figure 44. Response of isolated aortic rings to acetylcholine from normal and bile duct ligated rats ± lipoic acid drinking water. There was no difference in the vasodilation elicited by acetylcholine in isolated aortic rings from normal rats or BDL cirrhotic rats. Lipoic acid (LA)supplementation had no effect on acetylcholine mediated vasodilation in cirrhotic rats. Statistics carried out by two-way ANOVA (P=0.91). Normal sham n=3, cirrhotic rats n=6, cirrhotic rats + lipoic acid n=4.

5.4. Discussion

Previous studies from our laboratory have shown that lipoic acid (LA) supplementation can prevent the onset of the hyperdynamic circulation in the rat model of bile duct ligation[188]. It is known that isolated vascular tissue in BDL cirrhotic rats are hyporesponsive to stimulation with vasoconstrictors and this has been implicated in the hyperdynamic circulation. It was therefore speculated that the improvement in the hyperdynamic circulation following LA supplementation might be the result of an improvement in the integrity of the vascular tissue response to vasoconstrictors. However, isolated aortic rings from BDL cirrhotic rats did not respond differently to phenylepherine stimulation, whether treated with lipoic or not (Figure 43) and the creatinine clearance in those rats treated with LA was decreased compared to those given normal drinking water (Figure 40). Aortic rings isolated from rats treated with lipoic acid were equally hyporesponsive as those given normal drinking water compared to aortic rings isolated from non-cirrhotic rats. Although this result does not support the initial hypothesis, the vascular bed responsible for the majority of vasodilatation in the hyperdynamic circulation is the splanchnic bed not the aorta. Aortic rings were chosen in this study for ease of manipulation but do not necessarily represent tissue from the splanchnic bed. Experiments using mesenteric blood vessels would more accurately examine this.

Cirrhosis can lead to renal dysfunction (the hepatorenal syndrome), thought in cirrhosis to be secondary to the hyperdynamic circulation. It is known that creatinine clearance is

lower in the BDL cirrhotic rat than in normal rats[138]. However, whilst this study confirms our previous results and records an increase in plasma creatinine concentration in BDL cirrhotic rats (48μM sham – 70μM cirrhotic and 48.5μM sham - 62.4μM cirrhotic respectively), the creatinine clearance did not fall following bile duct ligation (Figure 40). The suitability of the 25-day model of bile duct ligation for investigation of the hepatorenal syndrome is therefore questionable. Interestingly though, whilst expecting that LA might improve renal function in BDL cirrhotic rats, LA supplementation actually decreased creatinine clearance significantly. The discrepancy in the expected and observed results was speculated to be the result of the pungent smell of lipoic acid. The BDL cirrhotic rats given lipoic acid drinking water did not consume or excrete as much liquid as those given normal drinking water which lends weight to this theory (Figure 41 and Figure 42). However, it is therefore hard therefore to distinguish the effects of lipoic acid from those of hydration in the lipoic acid treated animals. In future experiments examining the oral effect of LA, it might therefore be better to administer LA by gavage rather than in the drinking water.

Further investigations into the effects of lipoic acid on vascular dysfunction in cirrhosis were not carried out as preliminary *in vivo* haemodynamic experiments in anaesthetised rats were carried out following the above results (data not shown). The results of these experiments showed no effect of lipoic acid suplementation on splenic pulp pressure, blood pressure or renal blood flow in cirrhotic rats. This line of enquiry was therefore terminated and research efforts targeted towards the platelet/S-nitrosothiol studies discussed in chapters 2-4.

CHAPTER 6: DISCUSSION

Hypothesis

The initial aim of this work was to investigate the role of elevated S-nitrosothiols in vascular dysfunction and platelet aggregation in cirrhosis, and evaluate whether this dysfunction could be ameliorated by the use of low molecular weight antioxidants to reduce circulating S-nitrosothiol concentration. These hypotheses were based on previous studies showing the antiplatelet and vasodilatory properties of S-nitrosothiols at nanomolar concentrations *in vitro*[74] and *in vivo*[250] and highly elevated levels of S-nitrosothiols in the plasma of cirrhotic rats[217]. Furthermore, S-nitrosothiol half-life has also been shown to be reduced *in vitro*[250] following incubation with thiol antioxidants and *in vivo* following co-infusion with S-nitrosoalbumin[33]. Given that the predominant S-nitrosothiol found in the plasma is S-nitrosoalbumin, an analbuminaemic rat strain was also employed to further evaluate the role of S-nitrosothiols in liver disease.

It was noted that, upon induction of endotoxaemia in cirrhotic rats, S-nitrosothiol concentration was elevated dramatically above and beyond the rise found after induction of cirrhosis alone[217]. Endotoxaemia is also associated with increased variceal bleeding in cirrhotic patients[150]. It was therefore speculated that elevated plasma S-nitrosothiol concentration in cirrhosis with endotoxaemia may be implicated in abnormal platelet

function in these individuals and this was examined in the bile duct ligation rat model of cirrhosis. The potential therapeutic use of low molecular weight thiol antioxidants was also investigated.

Establishment of a model of cirrhosis in the analbuminaemic (NAR) rat

A BDL model of cirrhosis has been established and characterised in the analbuminaemic (NAR) rat and the progression of liver disease has been found to be equivalent to that in a normal, albumin synthesising, Sprague Dawley (SpD) rat strain, as assessed by histological and biochemical analysis (see chapter 3). Furthermore, NAR rats have been found to have comparable NO synthesis to SpD rats but to be protected against S-nitrosothiol formation due to the lack of the vicinal thiol on albumin. A comprehensive study into the aggregation profile of normal and cirrhotic analbuminaemic rats in response to ADP was compared to that in SpD rats and it was found that analbuminaemic rats are also protected against dysfunction in platelet aggregation in cirrhosis and endotoxaemia.

Establishment of a method for measuring total reduced thiol concentration in plasma

An adaptation to the classic Ellman's assay[243] has been made and validated for use in plasma to assess total reduced thiol concentration (see section 2.2.4.). The results of these studies have shown for the first time that total plasma reduced thiol concentration is markedly reduced in the BDL rat model of cirrhosis, probably due to the chronic conditions of oxidative stress.

S-nitrosothiols and vascular dysfunction in cirrhosis

Previous studies from our laboratory have shown that lipoic acid (LA) supplementation can prevent the onset of the hyperdynamic circulation in the rat model of bile duct ligation[188]. It is known that isolated vascular tissue in BDL cirrhotic rats are hyporesponsive to stimulation with vasoconstrictors and this has been implicated in the hyperdynamic circulation. It was therefore speculated that the improvement in the hyperdynamic circulation following LA supplementation might be the result of an improvement in the integrity of the vascular tissue response to vasoconstrictors. However, isolated aortic rings from BDL cirrhotic rats did not respond differently to phenylepherine stimulation whether treated with LA or not (Figure 43) and the creatinine clearance in those rats treated with LA was decreased compared to those given normal drinking water (Figure 40). Aortic rings isolated from rats treated with LA were equally

hyporesponsive as those given normal drinking water compared to aortic rings isolated from non-cirrhotic rats. Although this result does not support the initial hypothesis, the vascular bed responsible for the majority of vasodilatation in the hyperdynamic circulation is the splanchnic bed not the aorta. Aortic rings were chosen in this study for ease of manipulation but do not necessarily represent tissue from the splanchnic bed. Experiments using mesenteric blood vessels would more accurately examine this hypothesis.

Cirrhosis can lead to renal dysfunction (the hepatorenal syndrome), thought in cirrhosis to be secondary to the hyperdynamic circulation. It is known that creatinine clearance is lower in the BDL cirrhotic rat than in normal rats[138]. However, whilst this study confirms our previous results and records an increase in plasma creatinine concentration in BDL cirrhotic rats (48μM sham – 70μM cirrhotic and 48.5μM sham - 62.4μM cirrhotic respectively), the creatinine clearance did not fall following bile duct ligation (Figure 40). The suitability of the 25-day model of bile duct ligation for investigation of the hepatorenal syndrome is therefore questionable. Interestingly though, whilst expecting that LA might improve renal function in BDL cirrhotic rats, LA supplementation actually decreased creatinine clearance significantly. The discrepancy in the expected and observed results was speculated to be the result of the pungent smell of LA. The BDL cirrhotic rats given lipoic acid drinking water did not consume or excrete as much liquid as those given normal drinking water which lends weight to this hypothesis (Figure 41 and Figure 42). However, it is therefore hard to distinguish the effects of LA from those of hydration in the LA treated animals. In future experiments examining the oral effect of

LA, it might therefore be better to administer LA by gavage rather than in the drinking water.

Synthesis of mercury-stable NO-carrying species *in vitro* and discovery of it's anti-platelet activity

A mercury-stable NO-carrying species has been synthesised in plasma to probe the effects of the mercury-stable NO-carrying species (HgSNOCS) found in the plasma of endotoxaemic cirrhotic rats in this study. It was found that the HgSNOCS synthesised was albumin associated and that saturation of this type of nitrosation occurred when only 3% of available albumin was nitrosated. It is speculated that this species is a secondary N-nitrosamine due to the relative stability of the molecule in plasma and due to reports from others suggesting similar findings in normal rat[133] and human[251] plasma. Finally, it was shown that synthesised HgSNOCS have NO-like, but non-soluble guanylate cyclase mediated activity and can inhibit ADP-induced platelet aggregation when incubated with isolated human platelets. The NO dependence of this effect is reasoned from the available data but has yet to be proved. Experiments using an NO scavenger such as haemoglobin or detection using a fluorescence marker would test this hypothesis.

S-nitrosothiols

Since the previous studies carried out by our group into S-nitrosothiol concentration, other investigators have found that a significant portion of the signal recorded in normal plasma by the chemiluminescent method of detection of S-nitrosothiols was still detectable after pre-exposure of the plasma to mercury ions[132]. As S-nitrosothiols are unstable in Hg^{2+}, the relative stability of S-nitrosothiols in cirrhosis ± endotoxaemia was assessed. Whilst an increase in S-nitrosothiol concentration was recorded in cirrhotic rats ± endotoxaemia, it was found that the majority (74%) of the signal attributed in previous studies to S-nitrosothiols was in fact Hg^{2+}-stable and therefore not S-nitrosothiol (Figure 27). Interestingly, whilst the analbuminaemic rats used in this study to probe for the activity of S-nitrosothiols proved to have lower plasma S-nitrosothiol concentration, as expected due to their lack of albumin (Figure 26), they also demonstrated lower plasma mercury-stable-NO-carrying-species (HgSNOCS) generation following induction of cirrhosis ± endotoxaemia (Figure 27). It was also found that whilst injection of low molecular weight anti-oxidants, LA and NAC, had no effect on circulating S-nitrosothiol concentration in any conditions studied, it did lower the circulating concentration of HgSNOCS in endotoxaemic cirrhotic Sprague Dawley rats.

This contrasted with *in vitro* studies, where artificially synthesised HgSNOCS were exposed to thiols and were found to be stable (see section 5.3.5.2.). This suggests either that the synthesised HgSNOCS does not mimic the endogenous HgSNOCS, or that there is an active component to the stability of HgSNOCS *in vivo*. However, the stable

properties of the HgSNOCS formed by incubation of plasma with NO donors were otherwise similar to those found endogenously (a high degree of stability in the presence of mercury and resistance to decomposition in the presence of cyanide ruling out transition metal-NO species). Full characterisation of the species by structural analysis was beyond the scope and timescale of these studies but these initial results prove that this would be an interesting avenue for further research. An initial study was carried out to assess whether N-nitrosated albumin bound bilirubin could account for the HgSNOCS by exposing plasma from patients with varying degrees of hyperbilirubinaemia to the S-nitrosothiol, S-nitrosocysteine. However, the conditions under which this experiment was carried out did not represent saturating concentrations of nitrosation so the results were hard to interpret. Another possibility for identification of the N-nitrosated species is that proline is known to be susceptible to N-nitrosation[252]. An experiment in which a full protein digestion of nitrosated albumin and normal albumin with detection by GLC-MS[253] would determine whether albumin, or an albumin bound protein, may have a proline residue particularly susceptible to nitrosation.

The identity of the HgSNOCS is a particularly interesting direction, given the anti-platelet activity of the artificially synthesised HgSNOCS (see below). Analbuminaemic (NAR) rats were initially hypothesised to be conferred protection against platelet dysfunction in cirrhosis and endotoxaemia due to a lower potential for S-nitrosothiol formation under conditions of oxidative stress. However, given the higher molar abundance of plasma HgSNOCS compared to S-nitrosothiols in both Sprague Dawley and NAR rats, and the protection from HgSNOCS formation in NAR rats, the protection

against platelet dysfunction in these animals in cirrhosis and endotoxaemia could be equally attributed to the decreased HgSNOCS concentration. As a note of caution for further experiments, the injection of low molecular weight thiol antioxidants to endotoxaemic cirrhotic rats actually lowered the HgSNOCS concentration but did not affect platelet dysfunction. However, the reduction in HgSNOCS concentration conferred by NAC was only partial – 0.5μM HgSNOCS were still detected after NAC injection. Given the active element of HgSNOCS inhibition of platelet dysfunction (presumed given the stability of artificially synthesised HgSNOCS *in vitro* but not *in vivo* following exposure to thiols - see Figure 16 and section 5.3.5.2.), it could be that 0.5μM concentrations of HgSNOCS could confer maximal platelet dysfunction *in vivo*. This would be best assessed by concentration response infusion studies of HgSNOCS into rats (as in the studies of Orie et.al.[250]) and assay of platelet aggregation from platelets isolated from these rats.

Platelet aggregation

Relationship between endotoxaemia and platelet dysfunction in cirrhosis:

The dysfunction in platelet aggregation recorded following induction of cirrhosis in previous studies in human platelets[159,160,162] was also found here in platelets isolated from the bile duct ligated (BDL) cirrhotic rat (Fig.18). It was further shown that induction of endotoxaemia resulted in decreased platelet aggregation. However, for the first time a

correlation between endotoxaemia in cirrhosis and increased platelet dysfunction has been found (Fig.18). The platelet dysfunction in endotoxaemic cirrhotic Sprague Dawley (SpD) rats was profound, and was found to be greater than the sum of dysfunction observed in endotoxaemic rats and cirrhotic rats. This observation correlates strongly with the observation of increased risk of variceal bleeding in cirrhosis following bacterial infection[150]. Another study from our laboratory carried out simultaneously to these studies has shown that this pattern of impaired platelet aggregation in cirrhosis and endotoxaemia is paralleled in platelets isolated from human patients (Zambruni *et.al.*, submitted to publication).

The inhibition of platelet dysfunction in platelets isolated from either endotoxaemic or non-endotoxaemic cirrhotic Sprague Dawley rats was overcome by stimulation with supraphysiological (80μM) concentration of ADP. However, the profound dysfunction of platelet aggregation observed in the platelets of endotoxaemic cirrhotic Sprague Dawley rats could be only partially overcome by supraphysiological ADP stimulation. This, coupled with the observation that the initial rate of platelet aggregation in endotoxaemic rats was considerably lower than that of platelets isolated from controls, suggests that the mechanism of platelet dysfunction in endotoxaemic rats may be the result of impairment in the signal transduction cascade leading to granular release in platelets.

Mechanism of dysfunction in platelet aggregation

The mechanism of platelet dysfunction in endotoxaemic cirrhotic platelets was probed using the NOS inhibitor, L-NAME, and the guanylate cyclase inhibitor, ODQ. Neither incubation of platelets with L-NAME or ODQ affected platelet aggregation, ruling out platelet derived NO or an NO-cGMP dependent mechanism being responsible for the observed dysfunction. Any role for S-nitrosothiols/NO in the observed platelet dysfunction must therefore be through a cGMP independent mechanism. This is particularly interesting as the mechanism of GSNO inhibition of platelet aggregation has been demonstrated to signal predominantly through a cGMP independent mechanism[88]. A further experiment, which there was not time to complete in the process of these studies, would be to explore the findings of Sogo *et.al.*[89] who speculate that nitrosation of platelet ADP receptors could explain the anti-platelet cGMP-independent effects of S-nitrosothiols.

Low molecular weight thiol antioxidants failed to lower the plasma S-nitrosothiol concentration or affect platelet aggregation, so little can be deduced on the mechanism of platelet dysfunction in relation to S-nitrosothiols from these data. However, low molecular weight antioxidants did result in lower HgSNOCS (discussed later).

The establishment of a model of cirrhosis in an analbuminaemic strain of rats proved more informative and proved a correlation between raised S-nitrosothiol concentration in endotoxaemia and cirrhosis and decreased platelet aggregation (Figure 26, and Figure

31). Whilst the NO production in analbuminaemic rats was the same as in normal Sprague Dawley rats under all conditions studied, analbuminaemic rats formed lower concentrations of S-nitrosothiols than Sprague Dawley rats after induction of endotoxaemia and cirrhosis as expected. This also correlated to a reduced impairment of platelet dysfunction in analbuminaemic rats following induction of cirrhosis and endotoxaemia or induction of endotoxaemia alone. However, there was no difference in the platelet aggregation in analbuminaemic rats and normal Sprague Dawley rats following induction of cirrhosis without induction of endotoxaemia.

It is noteworthy that administration of low molecular weight antioxidants to endotoxaemic cirrhotic analbuminaemic rats resulted in severe haemorrhage in all 3 of these individuals and sudden mortality within 18 hours of administration at the 50mg/Kg dose administered. This was not expected as it had not been seen in any of the endotoxaemic cirrhotic Sprague Dawley rats studied, but is intriguing in light of the measurements of free thiol concentration in the plasma of normal and analbuminaemic rats with cirrhosis and/or endotoxaemia.

The plasma thiol concentration in healthy control analbuminaemic rats was very low compared to those in healthy control Sprague Dawley rats (27μM vs. 238μM free thiol respectively, Figure 24). This is presumably due to the near absence of albumin in these animals and therefore the single vicinal thiol on these proteins. Induction of cirrhosis in Sprague Dawley rats led to a fall in reduced thiol concentration to 60μM. Intriguingly, and conversely, induction of cirrhosis in analbuminaemic rats led to a rise in free thiol

concentration (27μM in sham operated rats vs. 64μM in cirrhotic rats), however, the total reduced thiol concentration was then similar in SpD and NAR rats. Although protein and low molecular weight free thiol concentration was not differentiated directly due to limited sample availability, these results may indicate that the chronic conditions of oxidative stress in cirrhosis leads to a dramatic decrease in free protein thiol concentration as evidenced by the results in SpD rats. However, the finding that free thiol concentration in NAR rats following induction of cirrhosis indicates that there is an upregulation of thiols from some source, presumably low molecular weight antioxidants being produced in response to the conditions of oxidative stress. Further studies examining the high and low molecular weight fractions of free thiol in cirrhotic rats could confirm this hypothesis, but there was insufficient sample volume to carry out these experiments.

Induction of endotoxaemia by injection of LPS resulted in a dramatic decrease in plasma free thiol concentration in both analbuminaemic and Sprague Dawley cirrhotic rats (27μM and 20μM respectively). However, as outlined above, injection of NAC only proved toxic in analbuminaemic rats. This differential reaction to NAC administration suggests that analbuminaemic rats could be a good model for probing the effect of thiol redox status in platelet aggregation in further studies. It suggests that key thiols may be required for correct platelet aggregation, especially as studies have shown that platelet surface thiols are involved in S-nitrosothiol mediated inhibition of platelet aggregation and binding of vWF to platelets[51,52].

The surprise finding of the platelet aggregation studies was that not only do analbuminaemic rats accumulate a lower concentration of S-nitrosothiols in the plasma following induction of cirrhosis and endotoxaemia, but also generate significantly lower plasma HgSNOCS concentration (Figure 27). Further, it was found that these HgSNOCS seem to be in the form of an albumin-NO adduct or albumin bound molecule-NO adduct and that this bond is postulated to be a secondary nitrosamine[133] (Figure 32 and Figure 33). However, whilst it is likely that NO is released or donated by this species and that this is an NO adduct, this has not been directly established. It is possible that the effects of HgSNOCS are independent of NO.

When thiol blocked human plasma was exposed to S-nitrosocysteine, it was found that the HgSNOCS species could be generated *in vitro*, and furthermore that it conferred anti-platelet activity in human platelets at the concentrations found in endotoxaemia and cirrhosis in rats (Figure 37). These results are particularly surprising given the known stability of the putative N-NO bond and the stability of these species in isolated plasma samples from endotoxaemic cirrhotic rats (no observed decomposition over 24 hours at room temperature). This suggests that there may be an enzymic mechanism of transfer of NO from the HgSNOCS against the platelet surface. A likely candidate for this transfer could be protein disulphide isomerase (PDI) on the platelet surface that has been shown to transfer S-nitrosothiol activity across the cell membrane[51]. Further studies into this mechanism were beyond the time limit and scope of this project but the evidence from these initial studies, especially considering the molar excess of HgSNOCS over S-nitrosothiols in the plasma of endotoxaemic cirrhotic rats, suggests that HgSNOCS are

potentially of equal interest as S-nitrosothiols in the mechanism of platelet dysfunction in endotoxaemia and cirrhosis.

Parallel experiments into platelet aggregation in platelets isolated from human cirrhotic patients that were carried out simultaneously in our laboratory have shown that the gross impairment of platelet aggregation in cirrhosis and endotoxaemia, demonstrated here in rats, is paralleled in platelets isolated from human infected cirrhotic patients (Zambruni *et.al.*, submitted for publication). However, the S-nitrosothiol and mercury-stable species recorded in humans was very low nanomolar (40nM in cirrhotic patients with infection) rather than the μM concentrations detected in rats (Figure 26 and Figure 27). These results suggest S-nitrosothiol/HgSNOCS are unlikely to be directly involved in the platelet dysfunction observed in endotoxaemia and cirrhosis in humans though it is possible that specific nitrosation of platelet ADP receptors as suggested by Sogo *et.al.*[89] could be involved. Again, dose-response infusion studies of S-nitrosothiols and HgSNOCS into the rat and subsequent sampling of platelet aggregation could test this theory.

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